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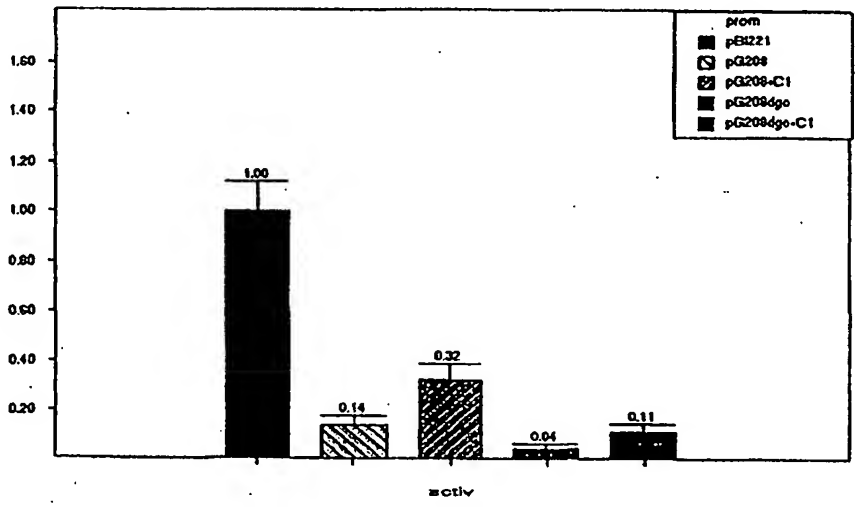

(57) Abstract

Disclosed are methods of activating a promoter derived from the V–sense promoter of a geminivirus (GV), which comprise the use of a transcriptional inducer associated with a nematode feeding structure or site (NFS). Particularly preferred are promoters derived from a monopartite GV or the A component of a bipartite GV. Also disclosed are variant promoters for use in these methods, methods for inducing NFS transcription of a desired nucleotide sequence (such as nematode control sequences) in plants, two component systems in which the promoters are activated by further inducers which are themselves controlled by NFS specific promoters, corresponding methods of reducing the susceptibility of plants to nematode infection, nucleic acid molecules and related processes for use in the methods of the invention, plus derived host cells and plants.

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INDUCIBLE PROMOTERS

TECHNICAL FIELD

The present invention relates to methods and materials for specifically expressing proteins and nucleic acid sequences in response to signals associated with endoparasitic nematode infection.

PRIOR ART

Nematodes

Plant endoparasitic nematodes can infect a great variety of plant species, from annual crops to fruit trees (Agrios, 1988). Since they constitute an important threat for many crop species, they are usually controlled by agrochemicals or through the use of natural resistance genes. Nematicides are expensive and often have a broad spectrum of toxicity which renders them potentially dangerous to animals and humans. In addition, resistance genes are not always available for a given crop, and even if they are, they are not always effective against nematodes under field conditions.

Nematode Feeding Sites and DNA synthesis

Endoparasitic nematodes are obligate biotrophs which induce specialized structures in the roots of parasitized plants, termed Nematode Feeding Sites or Nematode Feeding Structures (hereinafter NFSs) (Figure 1). NFSs are plant cells which have been modified by the nematode to fulfill its nutritional requirements: nematodes depend on NFS in an absolute way, since once the NFSs are formed, muscles in the nematode body degenerate and the animals become sedentary and linked to the NFS (reviewed in Sijmons et al., 1994a). If an NFS degenerates, the nematode cannot continue its development, and it dies before reproduction.

Two main groups of endoparasitic sedentary root nematodes can be distinguished by way of the type of NFS that they induce: cyst nematodes (*Heterodera* and *Globodera spp.*) and root-knot nematodes (*Meloidogyne* spp.). Cyst nematode NFSs are multinucleated

syncytia produced by the fusion of several adjacent cells; root-knot nematode NFSs are multinucleate giant cells formed through cell expansion and mitosis without cytokinesis (reviewed by Fenoll and del Campo, 1998)

NFSs are formed from pre-existing root prevascular cells by a cell differentiation process triggered by the nematodes, thought to be via signal molecules present in their salivary secretions (reviewed by Hussey, 1989). This differentiation process involves a first step in which DNA content increases by endorreduplication and, for root-knot nematodes, also by mitosis without cytokinesis. It has been demonstrated that cell cycle genes are activated by the nematode and that DNA synthesis occurs (reviewed by Gheysen et al, 1997), although the precise transcription factors responsible for NFS differentiation are unknown. Importantly many plant cells are known to enter endorreduplication pathways during differentiation (Melaragno et al., 1993; Grafi and Larkins, 1995), thus this effect *per se* is not peculiar to NFS development.

Use of NFS induced-promoters

NFSs are potential targets for controlling nematode parasites by means of recombinant DNA technology to inactivate the NFS or render it unsuitable for nematodes. However the extensive and/or strong expression of toxic or anti-NFS genes outside of the NFSs could produce undesirable effects on plant growth and development. Thus NFS-inducible promoters, with relatively high specificity, are desirable for such application.

It is known that, in addition to the DNA synthetic and endorreduplicative characteristics discussed above, during the NFS differentiation process there are changes in gene expression. It has been demonstrated that some of the changes happen at the transcriptional level. Several promoters have been described to be induced at these NFSs (reviewed by Sijmons, 1993; Fenoll et al., 1997).

WO 94/10320 (MOGEN INTERNATIONAL N.V.) discusses the use of DNA constructs containing promoters which are induced at initial nematode feeding cells, or NFSs, linked to a gene inhibitory to an endogenous (plant) gene. The exemplified promoter is that of the water channel protein TobRB7. Also discussed are methods for obtaining novel NFS inducible promoters. One method entails probing the plant genome using sequences based on mRNA which is highly transcribed at infected tissues. A different method employs interposon tagging of the plant genome using promoterless GUS constructs.

Opperman et al. (1994) discuss the possibility of expressing, specifically at NFSs, antisense RNA of proteins important for NFS development e.g. the water channel protein TOBRB7.

Fenoll et al (1997) discuss various genes which are induced at NFSs. Numerous plant-derived genes are listed. The authors briefly discuss the possibility of analyzing certain geminiviral promoters for nematode responsiveness. No details are given of which, if any, promoters were nematode responsive, nor whether these were specific for nematodes or were more broadly induced. The conclusions are concerned generally with plant promoters such as those identified in *Arabidopsis*. The possibility of dissecting such promoters for nematode responsive elements is raised.

It will be apparent from the foregoing, that the provision of novel promoter sequences which are nematode responsive, particularly those which have one or more advantages over those already available to the public, would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have shown that promoter sequences from certain geminiviruses are not only induced at NFSs, but that they are induced in a specific manner.

Geminiviruses (GVs) are small DNA viruses (reviewed by Lazarowitz, 1987) which code for proteins believed to drive the infected cells to the S-phase, in which DNA is synthesized and particular plant transcription factors are made available (Collin et al, 1996). GVs use a part of the cell machinery for their own replication and gene expression, which takes place by bidirectional transcription (Virion-sense and Complementary-sense, referred to as V- and C-sense) from a short and compact promoter. A number of GVs have been cloned and sequenced in the literature to date (over 60, including different strains). Most work done to date exploring GV promoter expression has been in dicotyledonous plants has been done with the dicot infecting geminiviruses Tomato Golden Mosaic Virus (TGMV) (Sunter and Bisaro, 1997) or Tobacco Yellow Leaf Curl Virus (Hong et al, 1996 Virology 20: 119-127).

The present inventors have also shown that a particular class of promoters (V sense promoters from GVs) are particularly effective in promoting specific transcription of linked protein sequences in NFSs. In order to achieve this they employed novel techniques based on transient expression by microinjection in NFSs.

Briefly, it has been shown that different geminiviral V-sense promoters from monocot and dicot infecting viruses (in particular, in the case of bipartite GVs, the V-sense promoter from the A component) are induced in various plant species including *Arabidopsis* at NFSs at moderate to high levels, and at early stages after infection. Certain elements of such promoters have been identified as having a possible role in this inducibility.

Interestingly certain other geminiviral promoters (e.g. C-sense promoters, and V-sense promoters from bipartite B components) showed no or only faint inducibility.

It had previously been assumed that *viral* proteins were absolutely required for V-sense transcription. Thus the

induction of V-sense promoters (which in the virus are linked to the production of coat proteins, not of proteins related to cell growth or cell metabolism) in the context of NFSs in plants is particularly surprising.

Further, the inventors have demonstrated NFS specificity in that a construct carrying the V-sense promoter from the monocot infecting Wheat Dwarf Virus (WDV) has been shown to be expressed in Arabidopsis NFSs whereas the same construct is not expressed in a suspension of dividing cells from wheat. This suggests a crucial difference in terms of transcriptional activities between NFSs and ordinary dividing cells which had not previously been appreciated in the art. Nor had any link between GVs and endorreduplication processes been demonstrated.

The inventors have also investigated the transcription factors and transcriptional activators which can under certain circumstances be used to activate these promoters and have shown that at least two proteins (WDV C1 protein Seq ID No.4 in Figure 2 and human E2F-1) when expressed recombinantly in plant cells can enhance GV promoter-driven gene expression in plant cells. Indeed the E2F observations by the present inventors may have broad applicability for transcriptional activation of plant or plant viral promoters, as could the transactivation of heterologous GV V-sense promoters by GV C1 proteins. Additionally the inventors have provided methods and materials based on the above findings which can be used, *inter alia*, to control nematode infestation in plants, for instance via the overexpression at NFSs of specific genes which interfere with nematode development and thereby generate plants with reduced nematode susceptibility.

These and other aspects of the present invention will now be described in more detail.

Thus in a first aspect of the present invention there is disclosed use of a transcriptional inducer associated with a NFS

for the activation of a GV V-sense promoter.

The term "inducer" as applied to the promoter describes a trans acting molecule, or particular combination of molecules, which occur naturally in an NFS, and which can stimulate (activate) expression which is operably linked (under the control of) the GV V-sense inducible promoter.

The terms stimulation, activation or induction in relation to the present invention are all used broadly, and cover the situations in which the inducer is employed to activate a GV V-sense promoter in:
(i) an essentially quiescent state in which there is low or undetectable levels of expression (or no expression) in the absence of the appropriate inducer,
(ii) a state in which there is detectable constitutive expression in the absence of the inducer, but that expression is increased in the presence of the inducer.

"Operably linked" will generally imply joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter.

By "NFS" is meant a nematode feeding structure or site (e.g. multinucleated syncytia or giant cells formed through cell expansion and mitosis) at any stage of development, from initial feeding cell onwards, provided that differentiation has commenced.

The NFS transcriptional inducer may be associated with any nematode-plant combination. For instance root knot nematodes (e.g. *Meloidogyne incognita*) or cyst nematodes (e.g. *Heterodera schachtii*). Lists of plant parasitic nematodes are given by Zuckerman et al (1971)(eds.) in: "Plant Parasitic Nematodes, Vol. I" pp 139-162, Pub. New York.

Similarly the invention is applicable to any V-sense promoter

capable of being so activated, e.g. those from monopartite viruses or the A component of bipartite viruses are particularly preferred. In preferred embodiments, where a highly specific response is required, the transcriptional inducer may be one associated with a dicot NFS, and this is used to activate a monocot-infecting GV.

Examples of suitable monocot virus promoters include those from Wheat Dwarf Virus (WDV, see Collin et al., 1996 - EMBL accession X02869) and MSV (Maize Streak Virus, see Fenoll et al., 1988 - EMBL accession K02026). An example of a dicot virus promoter is that from Pepper Huasteco Virus (PHV).

The promoter sequences from these viruses (WDV, MSV, PHV) are shown in Figure 2, and are designated Seq ID Nos 1, 2 and 3 respectively.

In one embodiment of the present invention, the use discussed above forms part of a method of inducing transcription from a nucleic acid comprising a nucleotide sequence operably linked to GV V-sense promoter, the method comprising causing or allowing the exposure of the promoter to a transcriptional inducer molecule associated with an NFS.

Such methods can be performed *in vivo*, both *in planta* and *ex planta* (e.g. using vectors in a suitable host cell). For instance the method may be employed in a test system for assessing the induction of a known, characterised, GV V-sense promoter with putative inducer molecules. Alternatively it may be used to compare the response of different GV V-sense promoters, or modified versions thereof, with known inducers. Examples of both of these systems are set out hereinafter.

However, preferably, the method is performed *in vivo*, i.e. the GV promoter is (stably) incorporated into a plant genome and the transcriptional inducer is present as part of the process by which an NFS is formed in that plant. Thus although the nucleic

acid encoding the GV promoter and appropriate coding sequence may be present systemically in the plant, transcription is induced locally at an enhanced level, in response to the formation of the NFS.

Thus in one embodiment the invention provides a method for inducing NFS-specific transcription in a plant of a nucleotide sequence, said method comprising use of a GV V-sense promoter. Particularly preferred is the use of a monocot GV V-sense promoter in a dicot plant.

The "specificity" (or specific inducibility) in this and other aspects of the present invention may be manifest as localised transcriptional activation of the promoter only at NFSs, or transcription which is enhanced with respect to other regions of the plant.

Control genes

One embodiment of this aspect of the invention provides a method of reducing the susceptibility of the plant to nematode infection thereby obviating the need for extensive use of nematicides, by hindering the induction, development or correct functioning of the NFS. This can be achieved, for instance, by the localised transcription of a nematode control nucleotide sequence operatively linked to a GV V-sense promoter.

By "nematode control nucleotide sequence" is meant one which, when transcribed, will inhibit the parasitisation of a plant by an invading nematode. Broadly speaking this may be effected by either directly acting on the nematode, or by inhibiting the formation of the NFS upon which it depends. As described in more detail below, such sequences may encode proteins, but there is no absolute requirement for them to do so.

Those skilled in the art will appreciate that even if absolute specificity is not obtained in a given instance, the methods of

the present invention which employ the NFS-activated promoters may still be advantageous over those employing constitutive promoters, particularly when controlling the expression of genes which may be potentially undesirable if expressed systemically.

Particularly preferred are sequences encoding nematicidal or cytotoxic protein, or which block the expression of plant genes important for NFS or nematode development or functioning, or which cause toxin-mediated NFS-suicide. Targeting house-keeping genes (i.e. those required for cell viability) in the NFS may be preferred.

For instance the GV V-sense promoters may drive the transcription of nucleotide sequences (e.g. of genes or fragments of genes) encoding any of the following: nematicidal proteins; antisense mRNAs for genes important for NFS functions; proteins or fragments of proteins from plants or other organisms which interfere with the induction or maintenance of NFSs; ribozymes against mRNAs from genes required at NFS; sequences capable of co-suppressing these genes.

More specifically the invention embraces nematode control sequences capable of any one or more of the following:

(i) Inhibition of nematode development by expressing, under the control of GV V-sense promoters, nematicidal proteins not toxic to plant cells or to humans and cattle. Proteins suitable for use in this embodiment may include collagenases, snowdrop lectin or other lectins, inhibitors of nematode proteases, neuropeptide antagonists, antibodies to secretions (for an example of the successful use of antibodies to alter plant phenotype see e.g. Plant J. (1995) 8: page 745)

(ii) Inhibition of nematode development by expressing, specifically at NFSs, cytotoxic proteins under the control of GV-V sense promoters (e.g. RIPs, Bt toxins, barnase, apoptosis-inducing proteins, ACC synthase to trigger ethylene synthesis,

enzymes for fitoalexin overproduction, enzymes capable of producing a hypersensitive response in the plant such as paired bacterial avirulence and resistance genes.)

(iii) Inhibition of nematode development by use of anti-sense versions of genes required for NFS development under the control of GV V-sense promoters.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of the promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. Antisense technology is reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

This approach is particularly effective when the expression pattern of the essential gene only overlaps at NFSs with the expression pattern of each the GV V-sense promoter used. Protein targets known to be present at NFSs, and which are thus candidates for antisense approaches, include: Lemmi9 from tomato; TobRB7 from tobacco; HMGRase, Histone 4A, cdc2aAt, cyc1aAt, rha1 from arabidopsis; ribulose phosphate epimerase (Favery et al, submitted), genes corresponding to tagged sequences inducible by nematodes (Barthels et al, 1997).

(iv) Inhibition of nematode development by expressing dominant negative versions i.e. mutant or truncated versions of proteins which lack the activity of the wild-type proteins but inhibit them in a competitive or non-competitive way (e.g. by competing for binding sites or receptors). Target proteins may be those which are crucial for NFS differentiation, expressed under the control of GV V-sense promoters e.g. E2F; RB; CDC2aAt

(v) Inhibition of nematode development by overexpressing under the control of GV promoters ribozymes directed against mRNAs coding for proteins essential to make a NFS, such as those

discussed above (for a review of ribozymes see e.g. Jaeger (1997) "The new world of ribozymes" Curr Opin Struct Biol 7:324-335, or Gibson & Shillitoe (1997) "Ribozymes: their functions and strategies form their use" Mol Biotechnol 7: 242-251.)

(vi) Inhibition of nematode development by overexpressing under the control of GV promoters a copy of all or part of the target gene inserted in a sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression. See, for example, van der Krol *et al.*, (1990) *The Plant Cell* 2, 291-299; Napoli *et al.*, (1990) *The Plant Cell* 2, 279-289; Zhang *et al.*, (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

The person skilled in the art will appreciate that specific transcription of sequences of the type discussed above may be more dependably achievable by using promoters not originating or derived originally from plant sources, since such plant-derived promoters may be more likely to be naturally activated at other regions and/or developmental stages in the plant. Thus GV V-sense promoters may be advantageous in this regard.

In order to achieve maximal specificity of transcription, the methods of the present invention preferably employ promoters derived from GVs which are not normally hosted by the plants in which the methods of the present invention are performed e.g. by using promoters from monocot infecting viruses in dicot plants.

Evidence that promoters from GVs known to infect monocot plants can be used in this way is presented hereinafter. This result is particularly surprising given that, generally speaking, monocot infecting geminiviruses are very different from dicot infecting ones. For instance the dicot ones are known to encode quite different proteins, none of which (with one exception, Tobacco yellow leaf curl virus) has the Rb binding domain. *Prima facie* this would suggest that most promoters from GVs known to infect monocot plants would not function (in any context) in dicot

plants.

As an alternative to a native GV V-sense promoter, a promoter which is a variant of a GV V-sense promoter may be used in the various aspects of the invention.

Generally this will share homology i.e. identity or similarity, with all or part of a native GV V-sense promoter, and will share NFS-inducible promoter activity as described above.

As used in relation to the various aspects of the invention discussed herein, unless context demands or suggests otherwise, the term GV V sense promoter covers also these variant promoters.

Changes, which may be by way of base substitution, deletion, or addition, may be desirable for a number of reasons, including introducing or removing restriction endonuclease sequences, or altering the length, strength, or specificity of the promoter with respect to the native promoter. For instance it may be desirable to remove motifs (e.g. the GC boxes in MSV, see Fenoll et al. 1988, 1990) which may bind transcriptional factors and thereby reduce specificity. An Example of such a modified promoter sequence is presented in pGus208dgc, as described in Example 8.

Variants may be prepared by those skilled in the art, for instance by site directed or random mutagenesis, or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly (e.g. via one or more amplification or replication steps) from an original nucleic acid having all or part of the native sequence (e.g. those shown in Seq ID Nos 1-3).

Specifically, variants may include promoters which have been extended at the 3' or 5' terminus.

Also included are fragments or other portions of the native sequences, however produced, having the requisite activity as

described above. For instance restriction enzymes or nucleases may be used to digest a nucleic acid molecule, or mutagenesis may be employed, followed by an appropriate assay (for example using a reporter gene such as luciferase) to determine the sequence required. Portions may also be isolated by use of specific primers to amplify selected motifs or other elements, for instance by PCR.

Fragments of interest include the conserved late element (CLE) disclosed in the Examples below, or, in the case of PHV, a 235 bp fragment.

Similarity or identity between the variant and the native promoter from which it is derived may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman.

Similarity or identity may be over the full-length of the relevant sequence, or may be over a part of it, preferably over a contiguous sequence of about or greater than 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267, 300 or more nucleotide bases.

Preferably, the variant shares at least about 50%, or 60%, or 70%, or 80% similarity, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% similarity with the native GV V-sense promoter.

Similarity may also be assessed by those skilled in the art, if preferred, by use of hybrisation screening. For example, hybridizations may be performed, according to the method of

Sambrook et al. (1989) using a hybridization solution comprising: 5X SSC (wherein 'SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):
T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

For suitably homologous promoter sequences, the level of activity may be quantified, for instance by using the transient expression methods discussed in more detail hereinafter, or methods analogous to those.

Activity can then be assessed by the amount of mRNA produced by transcription from the promoter, or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction.

Use of a reporter gene facilitates determination of promoter activity by reference to protein production. The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase, luciferase and green fluorescent protein (GFP). β-glucoronidase(GUS) and β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of GFP or luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes which may be used to determine promoter activity, for instance in conjunction with the transient assay

methods of the present invention.

Thus one aspect of the present invention comprises a method for assessing the NFS-responsiveness of a variant promoter comprising the steps of:
(i) preparing a nucleic acid construct comprising the variant promoter operably linked to a reporter sequence,
(ii) microinjecting the construct into an NFS, followed by
(iii) observing the NFS to detect transient expression of a reporter gene. The result of the observation may be compared with a control experiment carried out using non-NFS material.

However, generally speaking, any suitable reporter/assay may be used for assessing variants and it should be appreciated that no particular choice is essential to, or a limitation of, the present invention.

The compactness of the GV promoters, as compared with the much longer NFS-inducible plant promoters, renders them particularly suitable for fine dissection of discrete elements, and for carrying out deletion studies (e.g. to further improve NFS specificity).

Chimaeric promoters having the minimal elements or motifs responsible for NFS-inducible regulation, possibly in conjunction with other promoter sequences (e.g. taken from known plant promoters) form one part of the present invention. As described in more detail below, one apparently NFS-responsive element determined by the present inventors is the GV promoter CLE sequence, which makes a truncated 35S promoter active in NFSs. This finding is particularly surprising because the authentic 35S promoter is down-regulated at NFSs

The precise nature of the inducer responsible for providing the localised induction of GV V-sense promoters is as yet unknown. However those skilled in the art will appreciate that, provided that a given promoter is specific in the sense discussed above,

the invention may be performed even without this detailed information.

For instance the localised inducer which gives rise to the specific transcriptional activation of the native or variant (e.g. chimaeric promoter) may derive directly from a nematode (e.g. from a salivary or other secretion) whereby, in nature, it acts directly on endogenous plant promoters to promote NFS formation. Alternatively the transcriptional inducer may comprise a plant derived molecule, or battery of molecules, which does not occur elsewhere in the plant, but which plays a role in the development and differentiation of the NFS.

As described below an inducer may derive from neither the plant nor the nematode, but may be a heterologous molecule itself under the transcriptional control of an NFS-specific promoter. Thus the present inventors have determined that GV promoters may be activated, under certain circumstances, by the S-phase specific E2f transcription factor.

This interaction between E2F and GV promoters was previously unknown although there are published reports that WDV codes for a protein, C1, able to interact with RB (Collin et al., 1996). Others have published similar data for WDV involving different RB-type proteins (Xie et al., 1996; Grafi et al., 1996) and for a tomato-infecting virus, TMGV (Ach et al., 1997).

As described in the Examples hereinafter, the presence of E2F binding sites appears not to be a general feature of all GV V-sense promoters, or even of the subgroup I geminiviruses. Nor do E2F consensus sites appear to be widespread in plant promoter sequences. Thus their presence in WDV and MSV is particularly unexpected.

In one aspect of the present invention the findings regarding C1 and E2F may be exploited to increase the activation of the NFS-specific transcription. In particular the inventors have

provided a method for enhancing the induction of NFS-specific transcription in a plant of a first nucleotide sequence operably linked to an NFS-induced promoter, said method comprising use of an NFS-induced promoter operably linked to a second nucleotide sequence encoding a transcriptional inducer of the NFS-induced promoter.

This approach provides a "two component" system in which, for instance, a GV-V-sense promoter-anti-nematode fusion, is over-activated by a second fusion expressing an inducer (transcription factor, or transcriptional activator) of that promoter, under the control of that promoter. This effectively amplifies the initial transcriptional enhancement caused by the initial NFS-associated inducers.

Such an approach was not feasible using the various promoters described as being NFS-responsive in the published art because no suitable inducers had been characterised for them.

The first and second promoters will generally be the same. Preferably they will be GV-V-sense promoters (or variants thereof) in order to avoid undesirable expression of components. Indeed this approach will effectively amplify specificity, particularly if the first promoter is a variant promoter containing multiple transcription factor-binding sites while the second is a native GV-V-sense promoter.

Preferably the inducers will be selected from agents which are not naturally occurring in the host plant, for instance WDV C1 protein and E2F-1 (or analogs thereof capable of activating the promoter). The Examples below also show the use of mutant, modified, inducers based on C1 (see Example 9).

Interestingly, the present inventors have demonstrated that protein C1 from WDV activates a heterologous GV V-sense promoter (from MSV) in monocots and dicots. This implies a wide utility for this activation, for instance allowing the use of GV V-sense

promoters to detect C1 protein, or events associated with it. Similarly the discovery that GV-sense promoters are activated by E2F may be utilised by using the V-sense promoter (or part of it fused to a core mammalian promoter) to drive gene expression in animal cells in which E2F activity is high (such as tumour cells) of control sequences with the purpose of diagnosing them or ablating them.

In addition to the various methods and uses discussed above, in a further aspect of the present invention there is disclosed a nucleic acid molecule comprising a GV-V-sense promoter (or variant thereof e.g. a chimaeric promoter) operably linked to a sequence which it may be desired to express in a NFS-specific manner e.g. a control nucleotide sequence.

The various promoters and control sequences, particularly nematode control sequences, may be selected from any of those discussed above. In particular the constructs embodying the "two component system" are also included.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs (e.g. peptide nucleic acid). Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Nucleic acid molecules according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

The nucleic acid molecules may be wholly or partially synthetic. In particular they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run

contiguously) have been ligated or otherwise combined artificially. Alternatively they may have been synthesised directly e.g. using an automated synthesiser.

Thus in one embodiment in this aspect of the present invention, the nucleic acid may be in the form of a recombinant vector, e.g. a replicable vector. "Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host (or shuttle between the two) either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Suitable vectors, and appropriate host cells, can be readily chosen or constructed, containing appropriate regulatory sequences, including terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press or *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. Inasmuch as these references disclose more than the common general knowledge of the person skilled in this art, the disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Particularly of interest in the present context are nucleic acid constructs and vectors which operate in plants.

Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes such as resistance to antibiotics or herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

The present invention also provides methods comprising introduction of such a construct into a plant cell.

In a further aspect of the invention, there is disclosed a host cell containing a heterologous construct according to the present invention, especially a plant or a microbial cell.

The term "heterologous" is used broadly in this aspect to indicate that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention.

The host cell (e.g. plant cell) is preferably transformed by the construct, which is to say that the construct becomes established within the cell, altering one or more of the cell's characteristics and hence phenotype e.g. with respect to nematode resistance.

Nucleic acid can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in

Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei *et al.* (1994) *The Plant Journal* 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium alone is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Thus a further aspect of the present invention provides a method of transforming a plant cell involving introduction of a construct as described above into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce a nucleic acid according to the present invention into the genome.

The invention further encompasses a host cell transformed with nucleic acid or a vector according to the present invention (e.g comprising the GV-V sense promoter plus nematode control sequence) especially a plant or a microbial cell. In the transgenic plant cell (i.e. transgenic for the nucleic acid in

question) the transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. There may be more than one heterologous nucleotide sequence per haploid genome.

Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue, meristematic cell clusters, somatic embryos, or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Regenerated plants which include a plant cell according to the invention are also provided, along with any part thereof, seed, somatic embryo, clone, selfed or hybrid progeny and descendants and any part of these which includes the transformed cell. The invention particularly provides a plant propagule from such plants, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the

plant or an ancestor thereof.

The invention further provides a method of altering the nematode-resistance of a plant, the method comprising use of the nucleic acid constructs or vectors described above, optionally preceded by the earlier step of introducing them into a cell of the plant or an ancestor thereof.

Thus plants according to the present invention will have enhanced nematode resistance by virtue of the inducible nematode control sequences therein.

"Resistance" in this context means that the plants show a reduced susceptibility to parasitisation by a nematode as compared to a control plant. This may be assessed, for instance, by standard techniques (see e.g. Lamondia (1991), Plant Disease 75, 453-454; Omwega et al (1990) Phytopathol 80, 745-748). Those skilled in the art will appreciate that even embodiments of the invention which do not provide absolute resistance to nematode colonisation or predation, but instead simply reduce its severity, may still be commercially and practically beneficial.

The nature of the specific, localised manner in which the sequences are induced can reduce the possibility that the sequences (e.g. anti-nematode or cytotoxic proteins) may produce deleterious or other undesirable effects through being expressed elsewhere in the plant (e.g. at edible parts, or under special untested environmental conditions) or in other plant species (for instance following cross pollination in the field, for example). This may enhance the value of the plants from a regulatory or public perception perspective.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

FIGURES

Figure 1

This shows a schematic diagram of a generalised nematode feeding structure.

Figure 2

A. This shows the V-sense promoter sequence from the virus WDV (fragment Hpa-Hpa) used in pJIT39 (Seq ID No 1).

HpaII restriction site is shown in lower case (correspond to 2610 and 321 positions).

In bold face and underlined are marked nucleotides different to those of the WDV genome (x02869) from genebank database. They are:

Nucleotide	Position	Database Nt.	Database posn.
G	7	C	2656
T	85	A	2694
T	143	A	3
G	156	T	16
T	166	A	26
G	170	T	30
T	191	C	51
C	300	T	160
T	314	G	174
C	340	G	200 (in WDV2)

B. Sequence of MSV used in the Large Intergenic Region present in pGUS207 and pGUS208 (Seq ID No 2). The sequence introduced in pCF207 insert linkers xH01 (in position 2451 of the genome) and SaLI (in posisiton 2572 of the genome) is marked underlined, and

the nucleotides that differ from the published in genebank database (x01633) are marked in bold and underlined. They are:

Seq	Pos	Database	Pos
G	172	T	2009
G	646	A	2473

C. Sequence from PHV (Seq ID No 3).

D. The WDV-C1 protein sequence(Seq ID No 4)cloned intp pJIT163C1.

In bold and underlined are marked nucleotides and amino-acids which are different from the published in genebank database (X02869). They are:

seq	pos	aa	database	pos	aa
T	297	Asp	C	2297	Asp
C	344	Ala	T	2250	Val
C	605	Thr	G	1989	Ser

Figure 3

This shows the PHV-based constructs used in Example 1.

Figure 4

This shows the MSV- and WDV-based constructs used in Example 2.

Figure 5

This shows two of the constructs used in the preparation of transgenic tobacco plants:

A. pGreen 0029, constructed from pGreen 0000 as described in Example 3.

B. pGreen 0029:JIT39 containing the WDV promoter, constructed as described in Example 3.

Figure 6

This shows potential E2F-binding sites in the WDV Large Intergenic Region.

A. Diagram of the LIR from WDV (X02869). Coordinates in the circular genome are indicated by arrowheads. The hairpin loop conserved by all geminiviruses and implicated in replication is marked, so as the beginning of ORFs V1 and C1 which correspond to the virion- and complementary-sense transcription units, respectively. The orientation of the two E2F-like binding sequences, WDV-1 and WDV-2, are indicated by arrows, and their actual sequences shown below.

B. Comparison of the putative E2F-binding sequences with other known E2F binding sites. The two first sequences, defined as WDV-1 and WDV-2, come from WDV. The bases which differ from the consensus E2F1-binding site are marked in bold. The core sequence was defined by Kovesdi et al. (1986). The remaining sequences were defined in different E2F-responsive promoters transcribed either during the G1 to S transition or during the S phase of the cell cycle.

Figure 7

This shows a functional assay for the interaction of human E2F-1 with the WDV1 sequence and the maize RB protein.

A. Diagram of the reporter plasmids used in the One-Hybrid™ system. Oligonucleotides containing three tandem copies of WDV1 were inserted upstream form the promoters of the reporter genes *lacZ* and *HIS3*. Integration of both vectors into the yeast genome yielded the reporter yeast strain YSCi10.

B. Interaction of E2F-1 with the WDV sequence. YSCi10 was transformed with plasmids containing different fusions to the

GAL4 activation domain, and checked for growth on selective medium and for β-galactosidase activity. Transformants carrying a fusion to the human E2F-1 protein (pGAD424XhoIE2F-1) grew on the selective medium and showed blue colour in a filter lift assay, indicating an interaction between human E2F-1 and WDV1. No transformants could be selected after transformation of YSCi10 with plasmids carrying either a GAL4 fusion to the mouse p53 protein (pGAD53m) or the GAL4 activation domain alone (pGAD424XhoI).

C. Interaction of human E2F-1 with maize Rb. A yeast reporter strain (see Example 4) was transformed with a plasmid expressing a fusion of the GAL4 DNA-binding domain to a maize RB (pAS/zRb(RV-C)), plus a plasmid containing a fusion of the GAL4 activation domain the human E2F-1 (pGAD424XhoIE2F-1). Transformants tested positive for growth on selective medium and for β-galactosidase activity. The E2F-1 expressing plasmid only allowed residual growth of white colonies when co-transformed with a plasmid expressing H209, an inactive human RB (pASH209). Both the interactions of human wt RB with human E2F-1 (pASRb2 plus pGAD424XhoIE2F-1) and of maize RB with the WDV C1 protein (pAS/zRb(RV-C)) plus pACTC1:C2iNdeI) were positive.

Figure 8

This shows putative E2F binding sites in GVs.

Figure 9

Constructs used in Example 6.

A. The MSV- and WDV-based constructs used in testing promoter activity through bombardment into maize. Also shown is pBI221 which is based on the 35S CaMV promoter.

B. The corresponding effector constructs which express trans activators.

Figure 10

This shows the preparation of pJIT39 (Examples 3 and 6).

Figure 11

This shows the preparation of pJIT163C1.

A. Linear map of pStage IIIA in the region of the multiple cloning site. The open reading frames of WDV are shaded in plain grey (C1 and C2) or striped (V1 and V2). The tag at the end of the C2 coding region is indicated as a black rectangle. The pBluescript vector sequences are in bold lines.

B. Linear map of the WDV insert in pStage I
The construction of the plasmid is detailed in the text. The WDV open reading frames are in grey or striped as above. Bold lines either side of the insert are pSELECT™.

C. Linear map of the cloning region of pJIT163C1
The coding sequence of WDV C1 protein was cloned under the control of a double 35S promoter (grey rectangles). The WDV genomic sequence is indicated as a dashed rectangle, with the intron region shaded in black. The C1 STOP codon is also indicated. The whole sequence of pJIT163BgIII is not indicated but only represented as a bold line on both sides of the insert.

Figure 12

This shows the results of the transient expression experiments in maize described in Example 6.

Figure 13

A. This shows the MSV LIR in pGUS208 and flanking regions in pG208. The positions where the mutagenesis oligonucleotides bind are shown by arrows (see Example 8).

B. The sequence of the mutated LIR in pG208dgc.

Figure 14

Results of the transient expression of pG208dgc in maize leaves

as described in Example 8. The c1 experiments were carried out using pJIT163C1.

Figure 15

This shows the C1 coding region with the changed amino acid, the positions of the 4 oligonucleotides used to introduce the mutation, and restriction sites important for the cloning. The arrows mark the positions where the oligonucleotides OM1, OM2, OM3 and OM4 hybridise. The position where the mutation was introduced to make c1m is shown in bold and lowercase next to oligonucleotides OM2 and OM3 (G to A in the DNA sequence, E to K in the protein sequence). *HindIII* (AAGCTT) and *NdeI* (CATATG) sites are boxed in grey.

In bold and underlined are nucleotides and aminoacids (a.a.) different from those published in genebank database (X02869). They are:

Seq	Pos	a.a.	Database	Pos	a.a.
T	297	Asp	C	2297	Asp
C	344	Ala	T	2250	Val
C	605	Thr	G	1989	Ser

Figure 16

Results of bombardment and co-bombardment experiments were done in maize leaves using pJIT163C1m, an expression plasmid for the mutant C1 protein (c1m)

EXAMPLES

Example 1: Nematode induction of PHV promoter and chimaeric constructs in transgenic tobacco plants

Transgenic tobacco plants containing several fusions to *GUS* of promoters from the Pepper Huasteco Virus were obtained (see Ruiz-Medrano et al, 1999. Virology 253:162-169, although this publication is not essentially concerned with nematodes). Briefly, the sequences corresponding to the PHV shown in Figure 3 were excised from a clone containing the complete sequence of the

PHV (see Torres-Pacheco et al, 1993. J. Gen Virol 74:2225-2231) and inserted in a binary plasmid containing a convenient GUS reporter gene, by conventional molecular biology techniques. The binary plasmid was introduced in Agrobacterium, and the bacteria used to transform tobacco plants by conventional techniques. One line for each construct was selected to test nematode inducibility of GUS expression.

A scheme of the constructs is shown in Figure 3. Plants were grown in soil and infected with root knot nematodes (*Meloidogyne incognita*). GUS activity was evaluated at several points after infection (methods described in Aristizábal, 1996). The results of three independent repetitions are summarized in Table 1.:

Table 1.- GUS activity at NFSs in transgenic tobacco plants infected with *Meloidogyne incognita* at different times after infection.

Promoter-GUS fusion:	5 dpi	10 dpi	15 dpi	20 dpi
AR1 (693) (2 lines)	-	-	-	+
(235) (2 lines)	-	-	+	+
(115) (2 lines)	-	-	-	-
AL1	-	-	-	-
BR1	-	+/-	+/-	+/-
-90 35S CLE	+++	nd	nd	nd
-46 35S CLE	-	nd	nd	nd

(nd = not determined)

In summary: the C-sense promoter was not induced, and the V-sense in component B was only weakly induced. V-sense promoter from component A was the most active. Deletions that left only 235 bp of PHV LIR sequences 5´from the GUS gene ATG were active, but less than deletions leaving the whole LIR plus a fragment of AL1. Both truncated promoters were also active in non-infected plants very weakly in phloem of leaf and stem tissues, and almost silent in roots. Similar constructs made by Sunter and Bisaro (1997)

apparently showed some expression in phloem but not in actively dividing cells, such as meristematic cells. The lack of meristem expression accords with results from Tomato Golden Mosaic Virus (see Bisaro, 1997).

Both LIRs from A and B components have a conserved late element (CLE), also present in other GV, a proposed target for trans activation by the GV protein AL2 (Argüello-Astorga et al., 1994).

A construct harbouring a truncated -90 35S promoter flanked by a dimer of the CLE element was very responsive to nematodes at very short infection times. It also showed a temporally-related expression in some organs in non-infected plants, which did not appear to be associated with any particular organ or cell type. The CLE is a conserved element which occurs in 20 or 30 different GVs. For instance it occurs (in one or more copies) in the C subregion (from the end of the loop to the beginning of rightward transcription) of the bipartite geminiviruses (subgroup III) isolates from the old world in all members except in the A component of mungbean yellow mosaic virus (MYMV). It is present too in isolates from the New World like TGMV, SqLCV, PJV and PHV (see Argüello-Astorga et al., 1994).

In conclusion, it can be seen that a fragment of 235 bp containing the V-sense promoter from the A component of PHV is nematode-inducible. The C-sense promoter appears not to be inducible, while the V-sense promoter from the B component is slightly inducible. Since the C-sense promoter contains a CLE, it appears that the CLE *per se* may not be sufficient to confer NFS responsiveness.

However it also appears that the CLE can, in some contexts, confer strong and early nematode inducibility to a -90 35S promoter.

Example 2: Transient expression of GV promoters at NFSs in Arabidopsis

Transient expression of MSV and WDV promoter-GUS fusions were tested in NFSs using a novel microinjection technique based on that developed in NFSs induced by cyst nematodes in Arabidopsis roots (Bockenhoff and Grundler, 1994). The plasmids are shown in Figure 4.

The plasmids pMOG969 (Ohl et al., 1997) and HMG1-GUS (Lumbreras, 1995) were controls.

The pWDV4 plasmids are based on WDV and are disclosed in Collin et al. (1996). pWDV4iGUS cannot produce C1 and does not give GUS activity, or very little, in wheat cells. Plasmid pWDV3GUS can produce C1 but cannot replicate and does not give GUS activity in wheat cells either (Hofer et al, 1992).

PGUS207 and pGUS208 are based on MSV and can only produce truncated versions of C1. They were prepared by cloning the Hind III/Xba I fragment from pCF208 and pCF207 (Fenoll et al., 1988) in the plasmid pGUS1 from Plant Genetic Systems in the sites HindIII, Sal I, blunt-ended by fill-in reaction in the sites XbaI and SalI, 5' from the coding sequence of the GUS gene.

Plasmid DNA for microinjections was purified by using a commercial kit (Quiagen-tip 100), the DNA concentration adjusted to 0.7 μM and lucifer yellow 2 mM was added to visually follow the microinjection. From this solution, 10 to 100 pL were microinjected using a glass needle with a pore diameter of 0.2 to 0.5 micrometers into 8 days old NFSs, and roots were stained for GUS 24 to 48 hors later. GUS expression, when found, was restricted to the syncytium. The results are summarized in Table 2:

Promoter-Gus fusion	total injections	total positive NFSs	% positive NFS
pMOG969	43	1	2

HMG1 GUS	18	0	0
pWDV4GUS	21	3	14
pWDV4iGUS	31	4	13
pWDV3GUS	7	2	39
pGUS207	13	4	31
pGUS208	7	2	28

Table 2.- Expression of GV promoters microinjected in Arabidopsis NFSs induced by *Heterodera schachtii*

All constructs tested harbouring MSV or WDV promoters were induced, regardless of if they can produce viral proteins or not. Indeed, in spite of the differences, all constructs showed similar activities in NFS, indicating that V-sense expression must depend on transcriptional activities intrinsic of NFSs.

A control promoter known to be nematode-inducible (pMOG969 - see Ohl et al., 1997) was also positive (albeit at a low level) while other constructs gave negative results (such as HMG1-GUS, which is induced by root-knot nematodes but not cyst nematodes: unpublished data plus Lumbreras, 1995), showing that GUS expression depended only on particular constructs, and was not an artefact for the microinjection procedure.

Interestingly, pWDV3GUS, when bombarded in wheat cells (Hofer et al., 1992; Collin et al, 1996) did not show GUS expression, in spite of expressing the C1 protein; the fact that this construct is active in NFS indicates a crucial difference between dividing cells and NFS in the sense that *Arabidopsis* NFSs must have a transcriptional activity required for WDV V-sense expression that is not present in wheat dividing cells. Similarly pWDV4iGUS was expressed at only slightly higher than background levels in wheat cells (Collin et al, 1996) but gave clear induction in NFSs.

It thus appears that high levels of E2F-like plant factors, or of other transcription factors activated by C1, or unknown factors present at NFSs, must be crucial for activity. The WDV promoter

appears to be particularly NFS specific in that it is not expressed in dividing cells which are synthesizing DNA, which are likely to be the cells which most resemble NFSs in terms of transcriptional activity.

It can thus be seen that GUS-fusions based on the promoter from MSV (which infects monocots) are expressed in a dicot plant in NFSs induced by cyst nematodes. pWDV-based constructs, which have longer regions of the virus genome and code for viral proteins, can also be expressed in NFSs in a non-host plant. Importantly the V-sense WDV promoter is expressed in NFS, but not in dividing wheat cells, indicating that is not simply a promoter active in all cells in active proliferation and adding value to its application as an NFS-specific promoter.

Example 3: Preparation of transgenic nematode-resistant plants using GV V-sense promoters and variants thereof

General approach

Transgenes can be stably introduced in different plant species by conventional methods. The gene fusions to be transformed contain a selected GV V-sense promoter driving gene expression specifically at NFSs, the gene being capable of reducing NFS viability and hence inhibiting nematode development, leading to increased nematode resistance in the transgenic plant.

Firstly a GV V-sense promoter is selected, such as the WDV intergenic region present in pJIT39, and also a modified (variant) version of this promoter in which the original unique WDV-1 is replaced by a tetramer of the WDV-1 element (an E2F-1 binding site, see below). The activity of the promoters is tested by fusing them to the GUS reporter gene sequence, introducing the fusion into a binary vector and transforming Arabidopsis plants via Agrobacterium. Transgenic lines are selected as TRANSGENIC REPORTER LINES (see also Example 7) and the GUS expression pattern in non-infected plants and in nematode infected plants will be studied.

If there is little expression outside the NFSs, then the GV promoter may be used to drive the expression of a cytotoxin, such as the RIP dianthin, with the aim of producing cell death at NFSs. These protein have been shown to produce death in a cell-specific manner in other plant systems (for an example, see dianthin 30 used by Hong et al., 1996). Other proteins or parts of proteins with non-species specific cytotoxic effects may also be considered.

If the GV promoters show a degree of non-specific expression outside NFSs then the GV promoters may be used to drive the expression of antisense RNA for Arabidopsis genes which are expressed at NFSs, such as the HMG1 gene coding region, the sequence being obtained from the cDNA (Caelles et al., 1989 Plant Mol Biol 13: 627-638). The *HMG1* gene is highly induced by nematodes from very early during infection until completion of nematode development (unpublished), and the HMG1 protein may be necessary for NFS development (see Fenoll et al., 1997 for a discussion). It catalyzes the first step in the synthesis of isoprenoid compounds, such as sterols and other lipids, essential for membrane growth and therefore essential for NFS growth and development.

For both approaches, fusions of appropriate sense or anti-sense DNA coding sequences will be made by conventional PCR techniques to the promoter sequence in pJIT39. The fusions will be introduced in a binary vector, such as pBin19 or a similar plasmid, and transformed into Agrobacterium tumefaciens, which will mobilize the constructs into Arabidopsis plants. After selection of the transgenic plants, presence of the anti-nematode gene fusions will be confirmed by conventional methods, such as PCR. Several independent transgenic lines may be selected for further analysis e.g. observation of the whole plant life cycle, and nematode-susceptibility tests, to determine the relative decrease in susceptibility of the transgenic plants (PRIMARY RESISTANT PLANTS - see Example 7) as compared to wt Arabidopsis plants. The nematode species which will be preferentially tested

are *Meloidogyne spp.* and *Heterodera schachtii*. Naturally the above approach will be readily transferable to other plant species (particularly dicot plants) by those skilled in the art.

Examples of the use of WDV and MSV (natural and modified) promoters are set out below:

Material and in vitro cloning procedures

All in vitro recombination techniques employed were standard (Sambrook et al (1989) Molecular Cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press). Site directed mutagenesis was carried out using the pAlter system (Promega, Wisconsin) as recommended by the manufacturer. Sequencing was carried out using a dye terminator labelling procedure and employing an ABI 373A Automated Sequencing machine according to the manufacturer's instructions (Applied Biosystems, Foster City CA). The sequencing of pGreen is available on the EMBL/Genbank database and on the pGreen website (address given below).

All restriction and DNA modifying enzymes were from Boehringer Mannheim (Lewes, U.K.). All antibiotics were purchased from Sigma (St. Louis, Missouri).

pGreen vector

The pGreen vector is based on the general cloning vector pBluescript (Alting-Mees and Short, (1989) Nucl. Acids Res. 17, 9494) and therefore contains a colE1 *ori* for replication in *E. coli*. This plasmid's ampicillin resistance gene (encoding kanamycin resistance), the pSa *ori* was inserted, the f1 *ori* and Lac Z' region deleted and a Bg1II site was left for the introduction of a T-DNA cassette.

The T-DNA cassette, in the case of pGreen0000 is a 813bp Bg1II fragment including the pBluescript SKII LacZ' and multiple cloning site, synthetic LB and B sequences, derived from the

border sequences of PtT37 and with an additional T-DNA transfer enhancer ("overdrive") motif immediately adjacent and external to the RB (Slightom et al., (1985) EMBO J. 3, 3069-3077; Van Haaren et al, (1988) Plant Mol. Biol. 11, 773-781.).

Selectable marker and reporter gene cassettes

The basic pGreen vector contains no selectable marker or reporter genes for plant transformation. Internal to the T-DNA (LB and RB respectively) are unique HpaI and StuI sites for the insertion of selectable marker or reporter gene cassettes. Four selectable marker genes and two reporter genes have been modified by site directed mutagenesis, to remove all the restriction sites which would have been duplicated in the pGreen multiple cloning site. This includes the aph3'II (kan; resistance to kanamychin; Bevan et al., 1983) gene. The aph3'II gene does not contain the mutation that can affect its function as a selectable marker gene in some plant species (Yenifsky et al., (1990) Proc. Natl. Acad. Sci. UA. 87, 3435-3439). The function of the enzymes has not been affected by the DNA sequence changes introduced. The aph3'II gene coding sequence was fused to the nopaline synthase (nos) promoter-terminator and all extraneous sites at cloning junctions were removed.

pGreen 0029

pGreen 0029 was constructed by taking an EcoRV fragment harbouring nos: aph3'II (kansoph II); nos terminator into the Hpa I site of pGreen 0000 at the left border (see Figure 5A) using standard techniques. Nomenclature, from 1 to 728, was based on the use of a computer generated matrix to assign numbers for all the possible 35S and nos-containing cassettes cloned into the LB and/or RB cloning sites of pGreen0000.

pGreen 0029:JIT39

This is shown in Figure 5B.

For the preparation of pJIT39, standard in vitro recombination procedures were used throughout (Sambrook et al 1989). A 456bp HpaII fragment from WDV isolate CJI was recovered from pJIT34 (Woolston et al 1988). The fragment covered the Large Intergenic Region including the virion sense promoter (pV) from coordinates 2612-318 (circular genome size 2750bp; see Woolston et al 1988 for sequence). The fragment was treated with T4 DNA polymerase I to render the DNA ends blunt and the fragment was cloned into the Sma I site of M13mp18 (Yanisch-Perron et al 1985). Among recombinant clones the orientation of the WDV DNA was selected which had the pV promoter facing towards the HindIII site of the multiple cloning site in the M13 vector (see 1 in Figure 10).

To fuse the WDV pV promoter to the GUS reporter gene pJIT75 (see Figure 10; Guerineau and Mullineaux 1992) was cut with SmaI and KpnI and the larger plasmid DNA band was separated by agarose gel electrophoresis and eluted from the gel. The pV fragment in the M13mp18 phage was cut with PstI, T4 polymerase I treated and then cut with KpnI to make a blunt end KpnI fragment which could be cloned into the SmaI and KpnI sites respectively of pJIT75. The end-result is pJIT39 (see 2 in Figure 10) in which the pV promoter effectively replaces the CaMV 35S promoter.

A 2598bp Kpnl-Bgl II fragment from pJIT39 harbouring the WDV LIR pGV promoter fused to GUS with a CaMV 19 S terminator (poly A) sequence was inserted into the Kpnl-BamHI site of pGreen 0029 giving pGreen 0029:JIT39(map2), using standard techniques.

pGreen 0029:GUS208 and pGreen 0029:GUS208DGC

The preparation of pGUS208 is described in Example 2, and Figure 4. The modified DGC version is described in more detail in Example 8 and Figure 13. A 3708 bp HindIII-Xbal LIR MSV-GUS-ocs polyA fragment from pGUS208 was inserted into the Kpnl-BamHI site in pGreen 0029 creating pGreen 0029:GUS208, using standard techniques.

Plasmid handling and copy number in Agrobacterium

In Agrobacterium the pGreen plasmid requires the function of the RepA of pSoup (pJICSa_rep) to be maintained. pGreen contains no mobilisation function and so the plasmid is introduced into Agrobacterium using either electroporation or freeze-thawing.- A mixture of the pGreen plasmid and the pSoup (tetracycline resistant) can be used in a mixed electroporation. In this instance, selection for co-transformed Agrobacterium can be achieved by selection on kanamycin-containing medium only, since pGreen cannot replicate in Agrobacterium without pSoup being co-resident. Alternatively, electrocompetent Agrobacterium containing pSoup can be generated and re-electroporated with pGreen. A. *tumefaciens* strain AGL-1 supports pGreen replication provided that pSoup is also present. Thus A. *tumefaciens* strain AGL-1 harbouring pSoup and pGreen 0029:JIT39 OR pGreen 0029:GUS208 OR pGreen 0029:G208DGC can be transformed into tobacco using standard protocols, for example as generally described by Guerineau F., et al., (1990) Plant Molecular Biology 15, 127-136.

Analysis of plants

Following transformation, DNA was extracted from plants using the Plant Dneasy kit from Qiagen according to manufacturer's instructions.

10μG of DNA from each line was digested either with a) HindIII (GUS208 and GUS208DGC lines) or b) EcoRI (JIT39 lines). Standard Southern Blotting procedures according to Sambrook et al supra were used.

HindIII digests give plant DNA-TDNA border fragments of a minimum size of 5.5 Kb. Each band observed represented an independent integration event. 7 positive lines were observed for GUS208 transgenic lines ranging from single locus to >9 loci when probed with a GUS fragment. The GUS probe was prepared form a SmaI

1.8Kb fragment eluted from PJIT166 (Guerineau et al. (1992) Plant Mol. Biol. 18, pp 815-818) using standard procedures. In all lines these fragments were greater than the minimum size and therefore were likely to be intact genes.

For GUSDGC lines, 5 lines were identified ranging from a single locus to >5 loci. The same criteria apply as for GUS 208 lines.

EcoRI digests were performed on JIT39 lines. This gave an internal band of 3.3Kb (see Figure5B) when probed with ^{32}P-labelled GUS fragment. This was observed in 8 lines, ranging in copy number up to >10 copies.

The above data demonstrates that in all of the above lines the promoter GUS gene fusions were intact and unrearranged.

GUS expression in transgenic plants:

Transgenic tobaco seeds were provided as described above. In vitro-grown plants (1 to 3 weeks old) were stained for GUS activity. GUS activity was determined in whole plants directly in the Petri dishes as described by Jefferson (1987).

The results in the different lines:

pJIT39 (3 lines)
Roots: no expression
Stems and leaves: Weak expression in vascular tissue, mainly in stems. It is the stronger expresser, as compared with pG208 and pG208dgc.

pG208 (3 lines)
Roots: no expression.
Stems and leaves: Very weak in vascular tissue, mainly in stems. Occasional weak expression in stems at the sites of adventitious root emergence, where cortical tissue breaks as the roots emerge.

pG208dgc (3 lines)
Roots: no expression
Stems and leaves: Very weak in vascular tissue, mainly in stems.

Preliminary wounding experiments were done by pressing the leaves with flat forceps once, and incubating them for 30 minutes prior to GUS staining. A response was only detected in plants carrying pG208, with vascular tissue in petiols and leaves showing variable but clear GUS staining. Generally speaking, however, these experiments confirms that the promoters have a fairly low basal activity in these transgenic plants.

Methodology for inoculating transgenic plants with nematodes

Meloidogyne javanica WW4 was grown in hydroponic culture as described by Lambert *et al* (1992) Phytopathology 82: 43-46. Infective juveniles were collected by selecting actively moving nematodes through a paper tissue. These were counted under the microscope and used to inoculate plants. Juveniles were a kind gift from Francisca F. del Campo (University of Autonoma, Madrid, Spain).

For the expression pattern studies, transgenic tobacco plants were selected on kanamycin then transferred to pots containing sterilised soil and grown at 25°C/18°C (day/night) under a 16h light photoperiod for 1 week.

Nematode inoculation was carried out as follows. A suspension containing 1000 juveniles per ml of tap water was carefully pipetted through a wide-mouth pipette to minimise nematode shearing. A total of 0.5 ml of nematode suspension per plant (500 juveniles) were inoculated at a 1 cm depth in the soil immediately adjacent to the stem. After inoculation, plants were grown for 6 days, then carefully removed from the pots. The roots were washed with tap water to eliminate soil particles, and stained for GUS activity as described previously. After 12h staining, plant material was de-stained and observed under a

binocular microscope (Leika MZ6).

Results of nematode challenge

Three lines of each construct were examined.

pJIT39

Weak but clear gall expression was shown by one line of construct

pG208

For the pG208 lines examined, at the time and over the duration of testing, clear staining in galls was not detected.

pG208dgc

Clear GUS staining in the galls was observed in one line of the pG208dgc construct. In some cases this was clearly located on the inside of the gall, suggesting that the NFS itself was expressing the transgene as expected. On other occasions, GUS expression seemed broader in the gall, perhaps affecting the vasculature and the other tissues forming the gall.

Generally these results confirm the other results shown herein, namely that *M incognita* induced the PHV V-sense promoter in tobacco, and *H schachtii* feeding sites showed WDV and MSV V-sense expression when micro-injected into Arabidopsis.

Not all galls showed staining, an indication that promoter activation may be transient during the development of NFSs. This is not surprising, since NFSs differentiate over the course of several weeks, and it may therefore be expected that the geminiviral gene expression will vary over that time. Notwithstanding this, provided an appropriate control sequence is selected, there is no necessity that it be expressed over a sustained period.

Example 4: Regulation of GV V-sense promoters by cellular and viral proteins

The large intergenic region of WDV (LIR) was studied for E2F binding sites. This region contains the bidirectional promoter of the virus. Two potential E2F-binding sites were found on the complementary strand between positions 2742-2730 (WDV1) and 205-192 (WDV2) (Figure 6A). A comparison with functionally recognised E2F binding sites showed that the WDV sequences are not identical to the consensus sequence described for mammalian promoters (Figure 6B), but when comparison was made with the preferred binding site for E2F-1 (class I), the viral sequences showed only a 1 (WDV1) or 2 (WDV2) base pair mismatch. Since these changes in DNA sequence did not correspond to mutations known to abolish the binding of E2F, it was concluded that the sequences identified corresponded to functional E2F-binding sequences.

It should be noted that no E2F-binding sequences had previously been published in respect of plant promoters, nor had E2F protein(s) have been isolated nor their genes cloned from systems other than mammals. Of the two potential E2F binding sites, one of them, WDV1, was chosen for further study.

In order to assess the possible functionality of WDV1, its interaction with an E2F protein was tested using a genetic screen in yeast (the so-called "one-hybrid system") which consists of placing the sequence to be studied in the promoter region of two yeast reporter genes, whose expression becomes dependent on the interaction between the putative target sequence in the promoter and the DNA binding domain of the transcription factor that recognizes it. This DNA binding domain can be supplied as a clone of known sequence, fused to the GAL4 activation domain. In the present case the cDNA coding human E2F-1 was used. Although E2F proteins normally act as dimers with members of the DP family, they are also known to be recognisable on their own, albeit with a lower affinity. Thus it was anticipated that the GAL4/E2F-1 hybrid protein alone could bind to, and activate, the chimaeric promoters.

The experimental details were as follows:

The Matchmaker One-Hybrid System™ (CLONTECH) was used for testing the binding properties of the WDV putative E2F binding site, according to the manufacturer's protocols. The first step consisted in constructing the yeast reporter strain. A double-stranded oligonucleotide with the sequence 5'-AATTC(TTTTGGCGGGAGAA)$_3$G-3' corresponding to a tandem trimer of the WDV1 putative binding site was first cloned into *EcoRI*-digested BluescriptSK* to make pSC1H10. The insertion was sequenced to determine the orientation of the polylinker, and promoter region of the *lacZ* reporter gene in pLacZi, or with *EcoRV* and *SacI* for insertion in the promoter region for *HIS3* in pHiSi. The resulting plasmids were linearized, then used successively for transformation of the yeast receptor strain by integration into the genome. Screening of the different transformants was then carried out according to the manufacturer's instructions for selection of the clones with the lowest *HIS3* and *lacZ* background activity. The selected strain, pYSCi10, showed undetectable lacZ activity, and growth without histidine was residual in 25mM 3-Aminotriazole. This concentration was therefore chosen for further selection of the positive clones during the binding experiments. A map representing the orientation of the WDV1 sequences in the reporter gene promoters is shown in Figure 7A.

Fusion of the human E2F1 coding sequence to the GAL4 activation domain was carried out as follows. A *XhoI* linker (5'-AATTCCGCTCGAGCG-3') was first inserted between the *EcoRI* and *BamHI* sites in the polylinker region of plasmid pGAD424, which contains the GAL4 activation domain (AD); to construct pGAD424XhoI. This allowed the cloning of the E2F-1 cDNA sequence in frame with the GAL4 AD. This cDNA was excised from pCMV-E2F (Kaelin W.G., Jr. Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115, USA). The plasmid was first restricted at the 3'end of the cDNA with *EcoRI*, end blunted with T4 DNA polymerase, and then cut on the 5' side with *BamHI*. This fragment containing the full human E2F-1 cDNA was ligated into pGAD424XhoI previously restricted with *SalI*, made blunt, then

restricted with *BamHI*. The DNA sequence at the border between Gal4AD and E2F-1 is therefore : GAA TTC CGC TCG AGC GGA TTC CAT ATG GCC. The resulting plasmid is pGAD424XhoIE2F-1.

Transformation of the reporter yeast with pGAD424XhoIE2F-1 and screening of the yeast transformants was carried out as described in the manufacturer's instructions.

The YSCi10 reporter yeast strain was therefore constructed, and it contains the *HIS3* and *lacZ* genes placed under the control of the potential E2F binding site, a tandem trimer of WDV1 (Figure 7A). The tandem repeats are in the same orientation as transcription in *lacZ*, but not in *HIS3*, which shows the opposite orientation. This should not have a negative influence on E2F activity (and in fact both reporter genes showed E2F-dependent activation, see below) since several E2F-dependent promoters, such as the DHFR promoter, have one or more E2F binding sites in an inverted orientation (Blake et al., 1989). YSCi10 was transformed with a plasmid expressing the GAL4/E2F-1 hybrid protein (pGAD424XhoIE2F-1), and tested for its ability to grow on medium lacking histidine. The transformed strain was a histidine auxotroph (Figure 7B), which was an indication of a positive interaction between E2F-1 DNA binding domain and the WDV1 sequence. The interaction was further tested by monitoring in a filter assay the activity of the second reporter gene, *lacZ*, whose expression also turned out to be induced (not shown).

To test the specificity of the positive interaction between WDV1 and E2F-1, pYSCi10 was independently transformed with pGAD424XhoI, which is the plasmid coding for the GAL4 DNA activation domain but lacking the E2F-1 binding domain. This "empty" plasmid did not give a positive interaction, discarding the possibility of spurious GAL4 binding to WDV1 (Figure 7B). Another pGAD424-derived plasmid, coding for a fusion of GAL4 to the DNA-binding domain of the tumour suppressor protein p53, also failed to bind to WDV1, strengthening the conclusion that the interaction detected between WDV1 and the hybrid protein

GAL4/E2F-1 is specific of the E2F-1 moiety.

This hybrid protein also tested positive in a two hybrid system for interaction with the the maize RB protein (Figure 7C)., carried out as follows: The two hybrids assay was performed as previously described (Collin et al., 1996). Plasmids containing fusions of the GAL4 activation domain to the WDV C1 (pACTC1:C2iNde) (Collin et al., 1996) or the human E2F-1 (pGAD424XhoIE2F-1), where challenged with plasmids containing fusions of the GAL4 DNA-binding domain to wt (pASRb2) or a mutant inactive version (pASH209) of human RB (Durfee et al., 1993), or to a partial cDNA from maize RB that included both A and B pocket domains of the protein (pAS/zRb(RV-C)). The yeast strain and the transformation procedure were as described by Durfee et al. (1993). Screening of the yeast transformants was carried out as described in the manufacturer's instructions (Matchmaker library Protocol, kit PT1020-1, CLONTECH, USA).

Our positive results obtained suggest that plant E2F, if they exist, could interact with plant RB in a way similar to the one described for the mammalian proteins. This could explain why both the viral protein C1 and the human E2F-1 protein can trans-activate the E2F-binding site- containing WDV V-sense promoter, and suggests that C1 may operate via RB in vivo to activate V-sense transcription.

Example 5: Wheat and maize nuclear proteins that bind WDV1

The DNA fragment used in gel retardation assays was recovered from pSC1H10, digested with *EcoRI* and end-labelled by filling-in with [α^{32}P] dATP and Φ29 DNA polymerase 3'-5' Exo$^-$ (Bernad et al., 1989). The labelling reaction was conducted as described by Esteban et al. (1991). Gel retardation assays were performed essentially as described by Frieds and Crothers (1981). DNA used as non-specific competitor were synthetic oligonucleotides representing concatemers of the ACCGGGCCGG box that have been seen to bind specific proteins from maize extracts (Fenoll et

al., 1990). They were added to the binding mix prior to the nuclear protein extract.

By using this technique, nuclear extracts were made from actively growing wheat cell suspensions, and tested in electrophoretic mobility shift assays using as a probe the radiolabelled WDV1-trimer depicted in Figure 7A. The addition of wheat cell nuclear extract produced two clear bands flanking a fainter one, which appeared retarded with respect to the free probe. The faster migrating band appears at lower protein concentrations than the slower bands, more evident at higher protein content. This behaviour could be explained if the three bands represent DNA-protein complexes corresponding to occupancy of one, two or the three boxes present in the DNA fragment. Supramolecular complexes with additional proteins could also account for the lower mobility bands.

We also challenged the WDV1 trimer with nuclear extracts from maize, a monocot species related to wheat. Maize can be infected by the geminivirus MSV, which has a genome organization similar to WDV, also encodes a C1 protein with an RB-binding domain (Collin et al., 1996) and harbours E2F-like binding sites in the intergenic region. Nuclear extracts from maize cell suspensions showed a clear retarded band, which was weaker but detectable in tissues which had dividing cells, such as expanding leaves and root meristems from maize seedlings. Therefore, it seems that nuclear extracts from cells engaged in active division do have a WDV1-binding activity, as would be expected for an E2F-like activity. Equivalent amounts of total protein from nuclear extracts obtained from meristemless root-sections of the same plants, which have few cells in an active proliferative state, only had a residual binding activity (not shown). Maize protein extracts produced a diffused retardation in which a unique clear retarded band could be detected, coincident with the faster-migrating band obtained with the wheat extract. This could be explained if the maize proteins showed a reduced affinity for the heterologous DNA sequence, or lacked a putative synergistic

binding effect as compared to the homologous wheat proteins.

In order to assess the selectivity of the proteins for WDV1, competition experiments were performed as binding assays in the presence of large molar excesses of unlabelled oligonucleotides. Unlabelled WDV1 displaced the labelled WDV1 probe very efficiently from complexes made with both wheat and maize nuclear proteins, an indication that these proteins have a high affinity for the WDV1 sequence. However, equivalent amounts of a double-stranded oligonucleotide of similar size but with unrelated sequence, had little competitor effect. These results from the competition experiments indicate that protein(s) in wheat and maize cell nuclear extracts specifically recognize the E2F-like binding sequence of WDV1.

A search for 14 bp sequences with up to 4 mismatches with respect to defined E2F-binding sites, such that the general structure of E2F sites is conserved (a 5´stretch of 3 to 4 Ts, a 4 to 6 bases long G-C rich core and an A-T rich short 3´ end) allowed the identification of several potential E2F binding sites in various GV LIRs (Figure 8), at positions consistent with transcriptional regulatory roles. The presence of sites for putative E2F-mediated regulation is in agreement with the finding that many monopartite geminiviruses encode proteins with the consensus LeuXCysXGlu RB-binding motif (Collin et al., 1996), and that at least one bipartite geminivirus has been shown to encode a protein, AL1, with a confirmed interaction with RB (Ach et al., 1997).

Example 6: Use of transient expression in maize to test regulation of GV V-sense promoters in plants in presence and absence of effectors

It can be seen from the foregoing that WDV has two E2F-binding sites in its bidirectional promoter, and at least one of them (WDV1) can bind the human E2F-1 protein in a genetic test (yeast one-hybrid system), and can also bind nuclear proteins of plant origin (maize and wheat nuclear proteins) in vitro as detected by EMSAs, in a very specific way. Other GVs, such as MSV, also have

potential sites for binding of E2F-like proteins, and both maize and wheat cells have nuclear proteins which recognized WDV1 in a specific manner.

A series of reporter plasmids were prepared using the V-sense promoter from WDV and from the related maize-infecting MSV fused to GUS, plus also pBI221 containing the CaMV 35S promoter (Figure 9A; see also Jefferson, 1997). Two effector plasmids (based on E2F and C1) were also prepared (Figure 9B). Expression patterns following bombardment into leaves from 4 days old maize plants were studied as follows:

Maize seeds were germinated for 24 hours in the dark, over wet paper filter and under sterile conditions. Seedlings were subsequently grown for 4-6 days in a growth chamber under a 16 hours photoperiod, at 24 C. Leaves were collected and laid in a Petri dish with nutrient medium (MS, 3%sucrose 0.6 %agar) for bombardment.

Plasmids were prepared as follows:

(i) Construction of pJIT39 (see Figure 10).

See Example 3.

(ii) Construction pJIT163C1 (the double 35S-C1 plasmid)

All enzymes were from Boehringer Mannheim or New England Biolabs. The C1 coding sequence was isolated from plasmid pStageIIIA (Figure 11A). Pstage IIIA was made by successive cloning steps. Plasmid pStage I (see Figure 11B) was first made by cloning the WDV genomic sequence coding for part of C1 and the whole of C2 as an Ssti fragment isolated from pWDV2GUS (Gooding, P. S. 1995: PhD thesis, University of East-Anglia) into the SsTI cloning site of pSELECT™ (Promega). The C2 STOP codon was then mutagenized into an SphI site using the following oligonucleotide 5' -CCG CGC TAG GAC AGC ATG CTG CGA AGC AGT G-3'. A double-stranded

oligonucleotide (coding strand: 5' - TAC CCA TAC GAC GTC CCA GAC TAC GCC TGA-3') was then inserted into the blunt-ended SphI site of pStageI to make pMUTA, in which the C2 coding sequence has therefore been modified by addition of 9 extra amino acids at its C terminus.

In parallel, the NotI/XhoI fragment fro pWDV2 (Hofer, J.M.I., 1992: PhD Thesis, University of East-Anglia.) containing the whole of the C1 and C2 genomic sequence was subcloned into the NotI and XhoI restriction sites of phagemid pBluescript (Stratagene). The resulting plasmid was called pStage II. The wide-type NotI/HindIII fragment in pStage II was then replaced by the NotI/HindIII mutagenized WDV sequence isolated from pMUTA, to make pStageIIIA.

For expression of the C1 coding sequence under control of the 35S promoter, the C1 coding sequence was isolated as an NcoI/NdeI fragment (the coordinates in the WDV genomic sequences are 2590 and 1755, respectively) after the NdeI site was made blunt using T4 DNA polymerase. It was cloned into pJIT163BgIII (Creissen et al (1995) Plant J.8, 167-178) previously restricted with NcoI and SmaI to make pJIT163C1 (see Figure 11C). As a result of the cloning strategy, the NdeI site in the WDV sequence, as well as the SmaI site in pJIT163BgIII, are destroyed.

(iv) PJiT163E2F: E2F cDNA was excised from pCMV-E2F (Dana Farber Cancer Institute and Harvard Medical School, Boston, MA, USA - also as described in Cell 70:351-364, 1992, with the exception that the PCR mutation at residue 434 has been eliminated) and used to create pGAD424Xhol E2F-1, as described above.

E2F cDNA was EcoRI and HindIII digested from pGAD424XhoI E2F-1 and was ligated to pJIT163 previously EcoRI linearized. This originated a ligation product fusing the 35S promoter to the E2F cDNA. The molecule was made blunt, and both blunt ends were ligated to each other to obtain pJIT163E2F, carrying the E2F cDNA under control of a double 35S promoter.

DNA was coprecipitated with 1 mm diameter gold particles. Bombardments were made in a PDS100/He (Biorad) apparatus, at 1100 Psi . Bombarded leaves were kept under the same growth conditions for 24 hours, and stained for histochemical GUS assays were made by conventional procedures (Jefferson, 1987). Blue spots were counted from three independent experiments and the results statistically treated by one way ANNOVA.

Results from three independent experiments are shown in Table 3. The most prominent of these results are represented graphically in Figure 12.

Effector	none	2x35S C1	2x35S E2F1
Promoter-Gus fusion			
p207Gus	+	+++	
p208Gus	+	+++	
pJIT39	-	+++	++

Table 3.- Effect of C1 and E2F on transient expression of MSV and WDV V-sense promoters on maize leaves

These data demonstrate that in the absence of viral proteins expression from the GV promoters tested increases or depends totally on the presence of E2F.

The activity in the presence of C1 may be due to an ability to increase plant E2F-like activity through binding to RB. Expression in the absence of added E2F and C1, which may be due to promoter elements independent of the E2F sites, can be reduced by promoter deletions or other mutations. Modified promoters are tested for specificity as described above.

Since pJIT39 (based on WDV) is not active in young maize leaf cells, which are dividing, but can be activated by 35S-C1, it again seems that simply cell division or weak expression of C1

from its own promoter is not enough to induce the V-sense promoters strongly.

In other experiments (results not shown) pJIT39 was introduced into tomato (a dicot). Although, under the conditions used, the basal level of expression was higher than in maize, activation by C1 could still be detected. By comparison, pBI221 was not activated by C1.

Example 7 - Use of "two component" system

In order to enhance specificity and intensity of response, it may be desirable to use a two component system. One component may be a GV V-sense promoter (native or modified version) fused to the DNA sequence selected to confer nematode resistance (such as dianthin or anti-HMG1, or others) - this is the *nematode-resistance construct*; the second component is a fusion of the native GV promoter or other NFS inducible promoter, the expression pattern of which only overlaps with the GV promoter expression at NFS. This is fused to E2F-1 or to C1 (called *trans-activating construct*). Both components may be introduced into a plant host (e.g. Arabidopsis) via Agrobacterium, and nematode resistance is tested as described hereinbefore.

1. Construction of the *trans-activating plasmids*.

The WDV C1 and the E2F-1 sequences in plasmids pJIT163C1 and pJIT163E2F are fused by conventional techniques to the WDV intergenic region present in pJIT39. Both plasmids may be tested in the Arabidopsis TRANSGENIC REPORTER LINES constructed in Example 3, by microinjection in NFSs, to determine their effect as trans activators of GUS.

2. Construction of activator-expresser plants

If results of microinjection are positive, these trans activating gene fusions may be cloned into a binary vector and used to produce transgenic arabidopsis lines expressing the trans-activators under the control of the WDV V-sense promoter. These

TRANSGENIC ACTIVATOR LINES will be closely examined to rule out deleterious effects of C1 or E2F-1 expression outside the NFSs. If the plants are normal and fertile, they may be crossed with TRANSGENIC REPORTER LINES to determine if trans-activation leads to undesired GUS expression outside the NFSs.

3. If results are positive, TRANSGENIC ACTIVATOR LINES may be crossed with the PRIMARY RESISTANT PLANTS produced in Example 3. The resulting progeny will be screened for normal development plus enhanced nematode resistance.

4. If results in step 2 show deleterious effects of the activators expressed under the WDV V-sense promoter, then alternative promoters also active at NFS, but with an expression pattern which only overlaps with GV V-sense at NFSs may be used (see Fenoll et al., 1997; Barthels et al., 1997).

Example 8 - Promoter deletions in MSV

Construction of pG208dgc (promoter dissection of pG208)

pG208dgc contains a full length MSV LIR region as in pG208, but the GC boxes previously described (Fenoll et al., 1990) as essential for virion sense expression have been deleted. In principle such dissected promoters may have a lower basal expression in plant cells than the full length LIR, while maintaining responsiveness to specific activating factors, such as the C1 protein and other factors which may be associated with NFSs.

The GC box deletion was prepared by PCR using the following primers:

msv: CGGAATCATTTGCAACCACTATAAGACAAGGGAGCGG

msv loop: CGGAATTCAGCAGGAAAAGAAGGCGCGCACTAA

oligus: GATTTCACGGGTTGGGGTTT

m13reverse: CAGGAAACAGCTATGAC

The two oligonucleotides which hybridise with the sequences flanking the GC boxes, plus an additional tail with the EcoRI and two nucleotides to allow cloning, were termed msv and msv loop. We used two additional oligonucleotides flanking the MSV promoter, termed oligus, an oligonucleotide previously designed in this laboratory that hybridise with GUS coding sequence, and m13reverse, a comercial oligo (Boehringer) that binds sequences in pUC19 flanking the 5' end of the MSV LIR. EcoRI sites are underlined.

Figure 13A shows the MSV LIR in pGUS208 and flanking regions in pG208. The positions where the oligonucleotides bind are shown by arrows. Restriction sites important for the cloning are also marked. Bellow we show the PCR products A and B.

Using pGUS208 as a template, a first PCR reaction was carried out using oligonucleotides msv and m13reverse (PCR A), and another reaction (PCR B) with msvloop and oligus. The PCR A product was digested with HindIII/ EcoRI, and the PCR B product with NcoI/EcoRI, and the two fragments cloned at the same time into a backbone obtained by digesting pGUS208 with HindIII/NcoI. Positive clones were confirmed by sequencing the mutated fragment, and one of them was chosen as pG208dgc.

The sequence of the mutated LIR in pG208dgc is shown in Figure 13B.

DNA from the plasmid was obtained and used in bombardment experiments as described in Example 6.

Expression of pG208dgc in maize leaves

As shown in Figure 14, pG208dgc on its own has a very low activity, around 0.28 fold the activity of pG208.

However, the deleted construct still responded to the C1 protein from WDV, showing an increase over basal expression of 2.75x, similar to the increase experienced by the wild type promoter in pG208. These results demonstrate that the deleted MSV promoter, although very weak, can be activated by C1, the viral protein that interacts with the cellular retinoblastoma protein. Therefore, this deleted version is expected to respond to factors present in NSFs while having low background expression elsewhere in the plant.

Example 9 - Investigation of promoter induction by C1

Construction of pJIT163C1m, an expression plasmid for mutated C1

As described above, C1 interacts in yeast with RB, and may activate V-sense GV promoters by binding to cellular Rb and making it release transcription factors needed for V-sense promoter activation. To test *in planta* if the effect of C1 could be mediated by cellular Rb, we made a mutation in the Rb-binding motif of C1 (LICHE) changing E to K. This mutation has been demonstrated to abolish the interaction of Rep A (the C1 protein) with Rb (Xie, 1995).

We made this point mutation by PCR as described using standard procedures (Ausubel 1997), using oligonucleotides that have a mutation respect to the original sequence.

The oligonucleotides used are shown below. The mutations that change E to K in oligos OM2 and OM3 are marked in low case

OM1: CGTTGACGTCATATGTTGTGAAATCAACTA
OM2: CACTCATTTGCCATaAGACCATTGAAAGC
OM3: GCTTTCAATGGTCTtATGGCAAATGAGTG
OM4: CGTGCAGAACAAGCTTCGTGCTTCCATCACC

Figure 15 shows the C1 coding region with the changed amino acid, the positions of the 4 oligonucleotides used to introduce the mutation, and restriction sites important for the cloning.

PCR1 was prepared with OM4 and OM3, and PCR2 with OM1 and OM2 using as a template a plasmid containing the C1 sequence, pSTAGEIIIA (see Figure 11 A). A further PCR was carried out using as DNA template the combined products of PCR1 and 2, and the primers OM1 and OM4 . From this reaction a sequence representing a fragment of the C1 protein with a mutation that changes the codon GAG (that codes for E), to codon AAG (that codes for K) was obtained. This fragment was cut with HindIII and NdeI , cloned into the original site in pSTAGEIIIA restricted with HindIII and NdeI, and checked by sequencing. The modified pSTAGEIIIA was cut with NdeI, end-filled, and then cut with HindIII. The fragment was finally cloned in pJIT163 previously digested with HindIII/SmaI. One positive clone was selected as pJIT163C1m , an expression plasmid for the mutant C1 protein (c1m)

Mechanisms for C1-mediated GV promoter activation

Bombardment and co-bombardment experiments were done in maize leaves as described previously. The results obtained are represented in Figure 16

pJit39

Its very low basal activity was increased 26.5 fold by wild type C1 (pJit163), as described in Example 6. When the mutant c1m protein was used (pJit163C1m) the promoter was activated, but to a lesser extent (16.5 fold). This result indicates that a part of the C1-mediated activity maps to its interaction with the cellular RB, as discussed above. Therefore, the activation of WDV V-sense promoter by nematodes may in part be mediated by inactivation of RB in NFSs.

The results disclose that additional (unknown) domains in C1 also play a role in V-sense promoter activation independently from RB. Such domains may be investigated using further deletion-mutations of the C1 protein, for instance a deletion of a.a. 151 from one end.

pG208

Basal expression was enhanced 2.6 fold by C1 (pJIT163C1). However, when the mutated C1 protein was used (pJIT163c1m), there was no enhancement. This demonstrates that all the activation of the MSV V-sense promoter by the WDV C1 protein may be due to its interaction with the cellular RB protein (possibly via a release of E2F-like transcription factors needed for V-sense promoter activation), and no direct effect of the viral protein from WDV on the promoter is needed for transcriptional activation. Therefore the expression in NFSs of a C1-like factor, or combination of factors, which inactivate RB (possibly in conjunction with other activities) could release transcription factors and turn on the MSV V-sense promoter. Entry into the S-phase has been demonstrated (de Almeida Engler et al., 1999) to be needed at NFSs, and nematode secretions have also been found (Goverse et al., 1999) to induce cell division in plant protoplasts and mammalian cells - thus it is certainly feasible that nematode factors, in a direct or indirect way, act in this fashion. Additionally, the isolation of E2F-like transcription factors in plants has been recently reported (Ramírez-Parra et al., 1999), which is consistent with this view.

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Claims

1. A method of activating a promoter derived from the V-sense promoter of a geminivirus (GV), which method comprises use of a transcriptional inducer associated with a nematode feeding structure or site (NFS).

2. A method as claimed in claim 1 wherein the promoter is derived from a monopartite GV or the A component of a bipartite GV.

3. A method as claimed in claim 1 or claim 2 wherein the GV is selected from: Wheat Dwarf Virus (WDV) or Maize Streak Virus (MSV); Pepper Huasteco Virus (PHV).

4. A method as claimed in any one of the preceding claims wherein the promoter sequence is a wild-type GV V-sense promoter.

5. A method as claimed in claim 4 wherein the promoter sequence comprises any one of the following sequences:
(a) WDV promoter fragment (Hpa-Hpa) shown in Fig 2A (Seq ID No 1),
(b) MSV promoter LIR shown in Fig 2B (Seq ID No 2),
(c) PHV promoter region shown in Fig 2C (Seq ID No 3).

6. A method as claimed in any one of claims 1 to 3 wherein the promoter sequence is a variant of a wild-type GV V-sense promoter which:
(i) shares homology therewith, and
(ii) is activatable by a transcriptional inducer associated with an NFS.

7. A method as claimed in claim 6 wherein the variant promoter incorporates one or more deletions or substitutions with respect to the wild-type promoter

8. A method as claimed in claim 7 wherein the variant promoter comprises the modified MSV promoter sequence shown in pG208dgc of Figure 13B.

9. A method as claimed in claim 6 or claim 7 wherein the variant promoter is a chimeric promoter comprising all or part of a promoter derived from a GV V-sense promoter plus a second promoter

10. A method as claimed in claim 9 wherein the chimeric promoter comprises one or more conserved late elements of a GV V-sense promoter.

11. A method as claimed in claim 9 or claim 10 wherein the chimeric promoter comprises all or part of a CaMV 35S promoter.

12. A method for inducing transcription of a desired nucleotide sequence which is operably linked to a promoter derived from a GV V-sense promoter, the method comprising activating said promoter by a method as claimed in any one of the preceding claims.

13. A method as claimed in claim 12 wherein the desired nucleotide sequence operably linked to the promoter is present on a nucleic acid construct which is present in a plant.

14. A method as claimed in claim 13 wherein the promoter is derived from a monocotyledenous plant-infecting GV V-sense promoter, and the plant is a dicotyledenous plant.

15. A method as claimed in claim 13 or claim 14 wherein the nucleic acid construct is transiently present in the plant.

16. A method as claimed in claim 15 wherein the nucleic acid construct is selected from:
(a) pGUS208 as described in Example 2 herein,
(b) pJIT39 as described in Example 6 herein, or
(c) pGUS208dgc as described in Example 8 herein,

17. A method as claimed in claim 13 or claim 14 wherein the nucleic acid construct is incorporated into a plant genome.

18. A method as claimed in claim 17 wherein the nucleic acid construct is derived from pGreen0029 as described in Example 3 herein,

19. A method as claimed in any one of claims 13 to 18 wherein the transcriptional inducer associated with an NFS is present as part of the process by which an NFS is formed in the plant.

20. A method as claimed in any one of claims 13 to 18 wherein the promoter is activated by an activator transcriptional inducer which is encoded by a second nucleotide sequence.

21. A method as claimed in claim 20 wherein the activator transcriptional inducer is operably linked to a second promoter also derived from a GV V-sense promoter.

22. A method as claimed in claim 21 wherein the activator transcriptional inducer is selected from: WDV C1 protein, E2F-1 or analogs thereof.

23. A method as claimed in claim 22 wherein the activator transcriptional inducer comprises the c1m amino acid sequence shown in Fig 15A.

24. A method for inducing NFS-specific transcription in a plant of a desired nucleotide sequence, said method comprising use of a method as claimed in any one of claim 12 to 23.

25. A method of reducing the susceptibility of a plant to nematode infection by hindering the induction, development or correct functioning of the NFS or nematode by use of a method as claimed in claim 24 wherein the desired nucleotide sequence is a nematode control nucleotide sequence.

26. A method as claimed in claim 25 wherein the control nucleotide sequence encodes any of the following: a nematicidal or cytotoxic protein; an antisense mRNA specific for a gene required for NFS function; a protein or fragments of a protein which interferes with the induction or maintenance of the NFS; a ribozyme specific for mRNA from a gene required for NFS function; a sequence capable of co-suppressing a gene required for NFS function.

27. A method as claimed in claim 26 wherein the control sequence is an antisense sequence targeting any of: Lemmi9; TobRB7; HMGRase, Histone 4A, cdc2aAt, cyc1aAt, rha1; ribulose phosphate epimerase .

28. A method as claimed in any one of claims 25 to 27 for control of any of the following nematode species: *Heterodera, Globodera, Meloidogyne*.

29. A method as claimed in any one of claims 24 to 28 wherein the plant is a crop plant.

30. An isolated nucleic acid molecule for use in a method as claimed in any one of claims 25 to 29 comprising a promoter derived from a GV V-sense promoter operably linked to a nematode control nucleotide sequence.

31. A nucleic acid as claimed in claim 30 which is a recombinant vector.

32. A nucleic acid as claimed in claim 31 which is a plant vector.

33. A method comprising the introduction of vector as claimed in claims 32 into a plant cell.

34. A host cell containing a nucleic acid as claimed in any one of claims 30 to 32.

35. A host cell as claimed in claim 34 which is a plant cell.

36. A method of transforming a plant cell, the method comprising the method of claim 33, followed by the step of causing or allowing recombination between the vector and the plant cell genome to introduce the nucleic acid into the genome.

37. A plant cell transformed by the method of claim 36 such as to alter its phenotype with respect to nematode resistance.

38. A plant which includes the plant cell of claim 37, or any part, propagule, seed, somatic embryo, selfed or hybrid progeny or other descendant of said plant.

39. A method of altering the nematode-resistance of a plant, the method comprising use of a nucleic acid construct or vector as claimed in any one of claims 30 to 32, or the method of claim 36.

40. A method as claimed in claim 39 comprising the use of a two component system having:
(i) a first component which is a nucleic acid construct as claimed in claim 30,
(ii) a second component which is a nucleic acid construct comprising a second nucleotide sequence encoding an activator transcriptional inducer capable of activating the first component, and which is operably linked to a second promoter also derived from a GV V-sense promoter, the expression pattern of which overlaps with the first component at an NFS.

41. A method as claimed in claim 40 wherein the first component is introduced into a first plant to generate a primary resistant line, and the second component is introduced into a second plant to give a transgenic activator line, and the lines are crossed to give a plant having altered nematode resistance.

42. A method for assessing the NFS-responsiveness of a promoter sequence which is a variant of a wild-type GV V-sense promoter, which method comprises the method of claim 15 wherein,
(i) a nucleic acid construct comprising the variant promoter operably linked to a reporter sequence is prepared,
(ii) the construct is microinjected into an NFS,
(iii) the NFS is observed to detect transient expression of the reporter sequence.

43. A method for activating the expression of a control sequence in a mammalian cell in response to the E2F activity of that cell, the method comprising the steps of operably linking the control sequence to a promoter derived from the V-sense promoter of a geminivirus, and introducing the resulting construct into the cell.

44. A method as claimed in claim 43 wherein the cell is a tumour cell.

45. A method as claimed in claim 43 or claim 44 wherein the control sequence has the effect of ablating the cell.

Fig. 1A

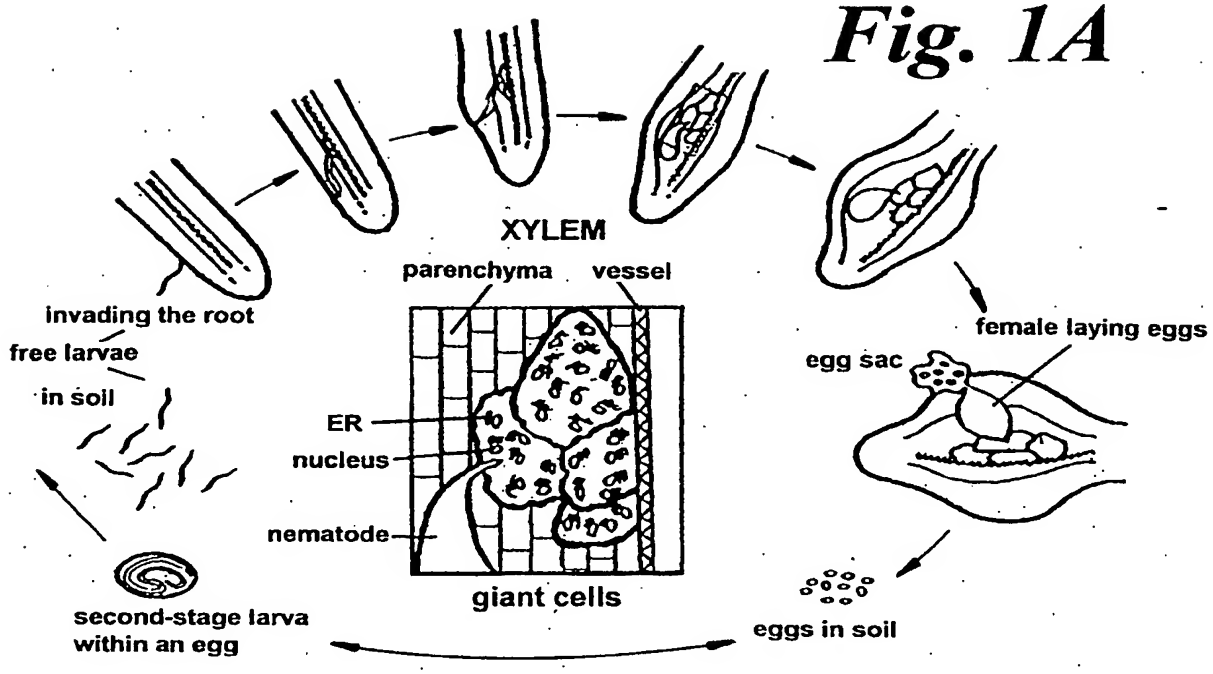
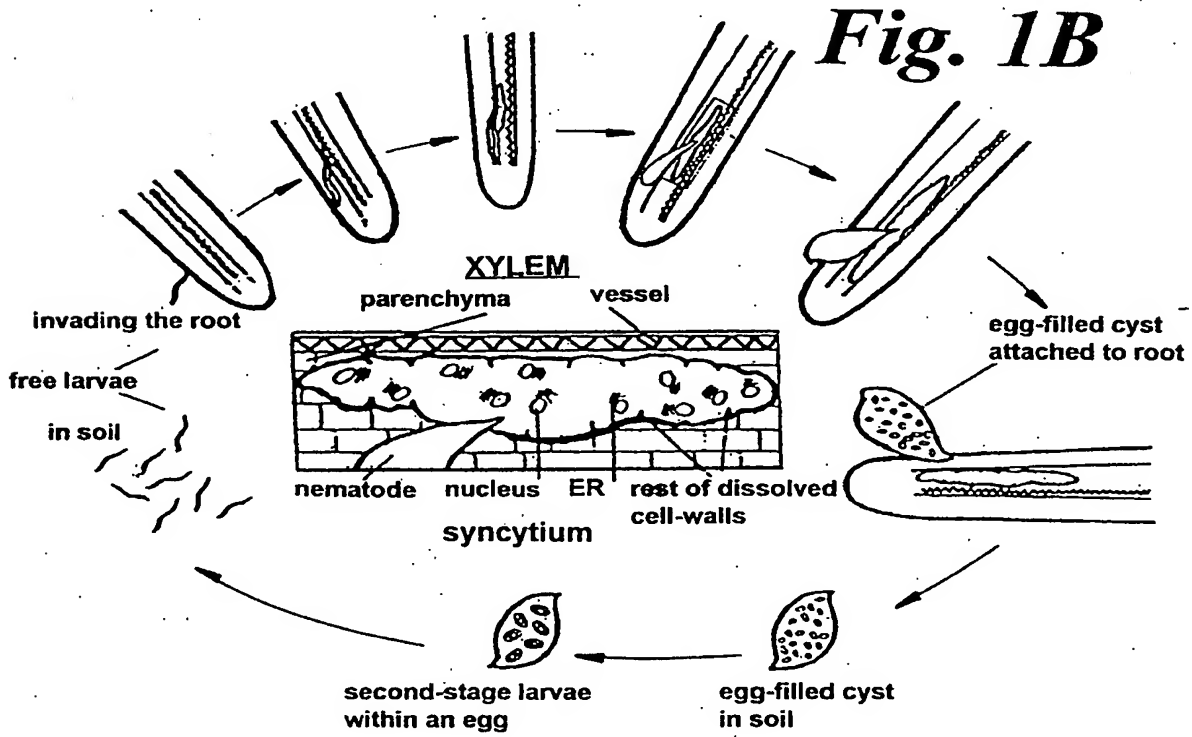

XYLEM
parenchyma
vessel
invading the root
free larvae
in soil
ER
nucleus
nematode
giant cells
female laying eggs
egg sac
second-stage larva
within an egg
eggs in soil

Fig. 1B


XYLEM
parenchyma
vessel
invading the root
free larvae
in soil
nematode
nucleus
ER
rest of dissolved
cell-walls
syncytium
egg-filled cyst
attached to root
second-stage larvae
within an egg
egg-filled cyst
in soil

```
        |    10      |    20      |    30      |    40      |    50      |    60
   1 ccggCAGGTC CTTAGCGAAA AAACGGGGTG TGCCAGAAAA CTCTATGCTC TACCCTGCGT 60
  61 GGAGGTGTGA ATTCTGCACA CTGCTAATGC AATGTGTCCA ATGCTTTATA TAGGGCAGGT 120
 121 TTTGGCGGGA GAACAGGGCC CTTGTGTTCC CACGGGAGCG TAGCGTATCG TGTGGGCCCT 180
 181 GTTCGGTGTG TGGTCGGGGG GCCTCCACGC GGGTTATAAT ATTACCCCGC GTGGTGGCCC 240
 241 CCGACGCGCA CTCGGCTTTT CGTGAGTGCG CGGAGGCTTT TGGACCACAT CTTTTCTGAC 300
 301 CACTTTCGTG GAATATGTTG ATTTATCACA CTTTTGACGC GGAAATCTGT GCCATGCCTT 360
 361 AGCTTATAAG GAAGTGCGTG GTAGCCCATC TCGATGGAGC AGGCAATAGC CCCCCCGCTT 420
 421 CCTATACGGG ACTATCAATA CCAGACCCCT TCCATTCccg g                     461
        |    10      |    20      |    30      |    40      |    50      |    60
```

Fig. 2A

```
        |    10      |    20      |    30      |    40      |    50      |    60
   1 AAGCTTATTT GCAGAGTATT CAAAATACTG CAATTTTGTG GACCAATCAA AGGGAAGCTC 60
  61 TTTCTGGATC ATGGAGAGGT ACTCTTCTTT GGAAGTAGCG TGTGAAATAA TGTCTCGCAT 120
 121 TATTTCATCT TTAGAAGGCT TTTTTTCCTT TACCTCTGAA TCAGATTTTC CGAGGAAGGG 180
 181 GGACTTCCTA GGAATGAAAG TACCTCTCTC AAACACAGCC AGAGGTTCCT TGAGAATGTA 240
 241 ATCCCTCACC CTGTTTACTG ACTTGGCACT CTGAATATTT GGGTGAAACC CATTTATATC 300
 301 AAAGAACCTT GAGTCAGATA TCCTTACCGG CTTCTCTGTC TGAAGCAATG CATGTAAATG 360
 361 CAAACTTCCA TCTTTATGTG CCTCTCGGGC ACATAGAATG TATTTGGGAA TCCAACGAAC 420
 421 AACGAGCTCC CAGATCATCT GACAGGCGAT TTCAGGATTT TCTGGACACT TTGGATAGGT 480
 481 TAGGAACGTG TTAGCGTTCC GGTGTGAGAA CTGACGGTTG GATGAGGAGG AGGCCATTGC 540
 541 CGACGACGGA GGTTGAGGCT GAGGGATGGC AGACTGGGAG CTCCAAACTC TATAGTATAC 600
 601 CCGTGCGCCT TCGCCTCGAG GCGAAATCCG CCGCTCCCTT GTCTTGTAGT GGTTGCAAAT 660
 661 GGGCCGGACC GGGCCGGCCC AGCAGGAAAA GAAGGCGCGC ACTAATATTA CCGCGCCTTC 720
 721 TTTTCCTGCG AGGGCCCGGT AGGGTCGACC CCGAGCGATT TGATGTAAAG TTTGGTCCTG 780
 781 CTTTGTATGA TTTATCTAAA GCAGCCCATT CTAAAGAATC CGGTCCCGGT CACTATAAAT 840
 841 TGCCTAACAA GTGCGATTCA TTCATGGATC CACAGAACGC CCTGTATTAT CAGCCGCGGG 900
 901 TACCCACAGC AGCTCCGACA TCCGGAGGAG TGCCGTGGAG TCGCGTAGGC GAGGTAGCTA 960
 961 TTTTGAGCTT TGTTGCATTG ATTTGCTTTT ACCTGCTTTA CCTTTGGGTG CTGAGAGACC 1020
1021 TTATCTTAGT TCTGAAGGCT CGACAAGGCA GATCCACGGA GGAGCTGATA TTTGGTGGAC 1080
1081 AAGCTGTGGA TAGGAGCAAC CCTATCCCTA ATATACCAGC ACCACCAAGT CAGGGCAATC 1140
1141 CCGGGCCATT TGTTCCATCG ACTCTAGTCG ACC                              1173
        |    10      |    20      |    30      |    40      |    50      |    60
```

Fig. 2B

```
CATATTTGTA ATAAGAGAGG TGTACACCGA TTGGAGCTCTTTAACCTGGG
CTTATTGTAT CGGTGTATTG GTAGCCAATA TATAGTATATGGGAGTTATC
TAGGATCTTC GTACACGTGA GGGCCATCCGT TATAATATTA CCGGATGGCC
GACCGCTTAC CTTATCTATCCGTACTGCTT TATTTGAATT AAAGATGTTA
CTTTTATGCT ATCCAATGAAGCGTAGCGTC TGGGAAGCTT AGTTATCAGT
TCCAGACGTG GGGACCAAGTAGTGTATGAC CACTTTATTG ACTGTCAGCT
TTATAAATTG AAATTAAAACATAAGTGGTC CATGTACCTT TAATTCAAAA TG
```

Fig. 2C

Figure 2D

```
1/1                                                                 31/11
ATG GCC TCT TCA TCT GCA CCC AGG TTC CGT GTC TAT TCC AAG TAC CTC TTT CTA ACA TAT
M   A   S   S   S   A   P   R   F   R   V   Y   S   K   Y   L   F   L   T   Y
61/21                                                               91/31
CCT GAA TGT ACC CTT GAG CCA CAG TAC GCC TTG GAT TCA CTT CGC ACT CTC TTG AAC AAA
P   E   C   T   L   E   P   Q   Y   A   L   D   S   L   R   T   L   L   N   K
121/41                                                              151/51
TAT GAG CCC CTC TAC ATC GCT GCT GTT AGA GAG CTC CAC GAA GAT GGA TCA CCA CAT CTG
Y   E   P   L   Y   I   A   A   V   R   E   L   H   E   D   G   S   P   H   L
181/61                                                              211/71
CAC GTT CTC GTG CAG AAC AAG CTT CGT GCT TCC ATC ACC AAT CCC AAT GCC TTA AAC CTC
H   V   L   V   Q   N   K   L   R   A   S   I   T   N   P   N   A   L   N   L
241/81                                                              271/91
CGT ATG GAT ACA TCT CCA TTC TCC ATA TTC CAT CCA AAT ATA CAA GCT GCC AAA GAT TGC
R   M   D   T   S   P   F   S   I   F   H   P   N   I   Q   A   A   K   D   C
301/101                                                             331/111
AAC CAA GTT CGT GAT TAC ATC ACG AAG GAG GTT GAC TCC GAT GCA AAC ACA GCT GAG TGG
N   Q   V   R   D   Y   I   T   K   E   V   D   S   D   A   N   T   A   E   W
361/121                                                             391/131
GGA ACA TTC GTG GCT GTT TCA ACT CCA GGT CGT AAA GAC CGT GAT GCG GAT ATG AAA CAG
G   T   F   V   A   V   S   T   P   G   R   K   D   R   D   A   D   M   K   Q
421/141                                                             451/151
ATC ATT GAA TCT AGT TCC TCT CGC GAG GAA TTC CTC AGC ATG GTT TGC AAT CGT TTT CCG
I   I   E   S   S   S   S   R   E   E   F   L   S   M   V   C   N   R   F   P
481/161                                                             511/171
TTT GAA TGG TCT ATC CGT CTC AAA GAC TTC GAG TAC ACG GCA CGC CAT CTA TTT CCT GAC
F   E   W   S   I   R   L   K   D   F   E   Y   T   A   R   H   L   F   P   D
541/181                                                             571/191
CCA GTT GCC ACT TAC ACA CCT GAG TTT CCA ACC GAA TCA CTC ATT TGC CAT GAG ACC ATT
P   V   A   T   Y   T   P   E   F   P   T   E   S   L   I   C   H   E   T   I
601/201                                                             631/211
GAA ACC TGG AAA AAT GAA CAT CTC TAC TCC GTA AGC CTC GAA TCC TAT ATC CTT TGT ACT
E   T   W   K   N   E   H   L   Y   S   V   S   L   E   S   Y   I   L   C   T
661/221                                                             691/231
TCC ACT CCT GCG GAT CAA GCG CAA TCT GAC TTA GAG TGG ATG GAC GAT TAT TCC AGG AGT
S   T   P   A   D   Q   A   Q   S   D   L   E   W   M   D   D   Y   S   R   S
721/241                                                             751/251
CAC CGG GGA GGC ATA AGT CCA TCT ACA TCT GCG GGC CAA CCA GAA CAG GAA AGA CTT CCT
H   R   G   G   I   S   P   S   T   S   A   G   Q   P   E   Q   E   R   L   P
781/261
GGG CAA GGT CTC TAG
G   Q   G   L   *
```

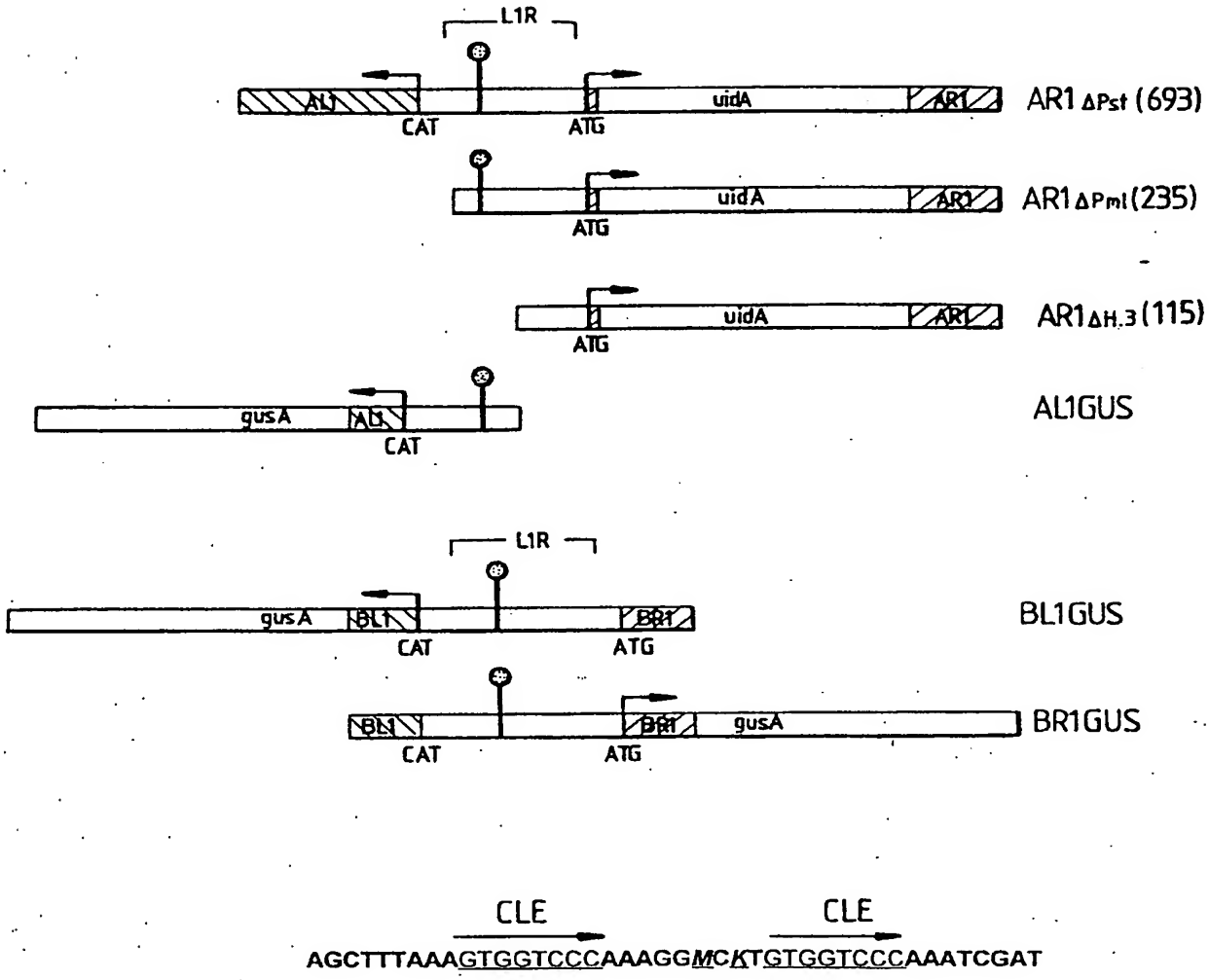
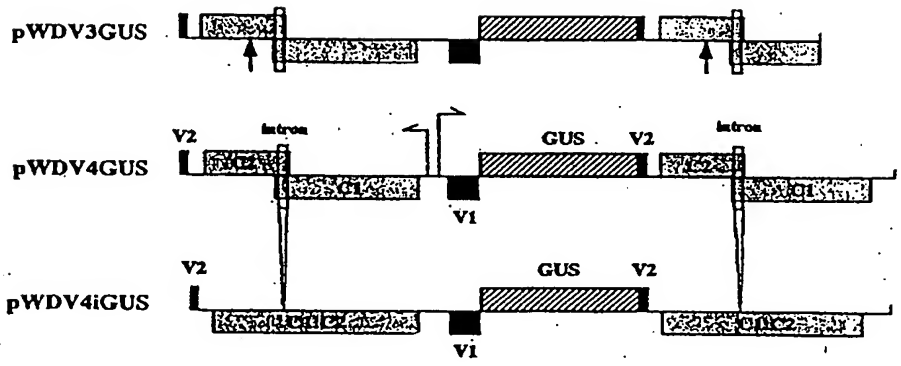

L1R
AL1
CAT
ATG
uidA
AR1
AR1 ΔPst (693)
AR1 ΔPml (235)
AR1 ΔH.3 (115)
gusA
AL1GUS
BL1
BR1
BL1GUS
BR1GUS
CLE
CLE
AGCTTTAAAGTGGTCCCAAAGGMCKTGTGGTCCCAAATCGAT
CLEs
−46 (35S)
−90 (35S)
Δ46CLE
Δ90CLE

Fig. 3

Figure 4


pWDV3GUS
pWDV4GUS
pWDV4iGUS
V2
Intron
GUS
V2
Intron
V1
V2
GUS
V2
V1

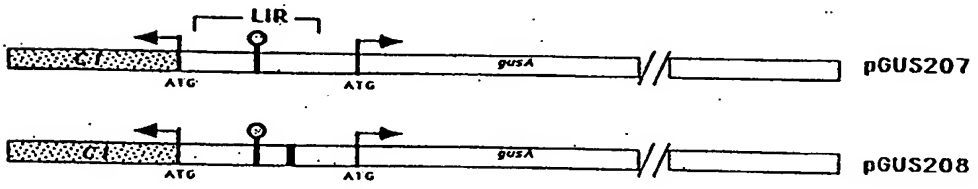

LIR
ATG
ATG
gusA
pGUS207
ATG
ATG
gusA
pGUS208

Figure 5A

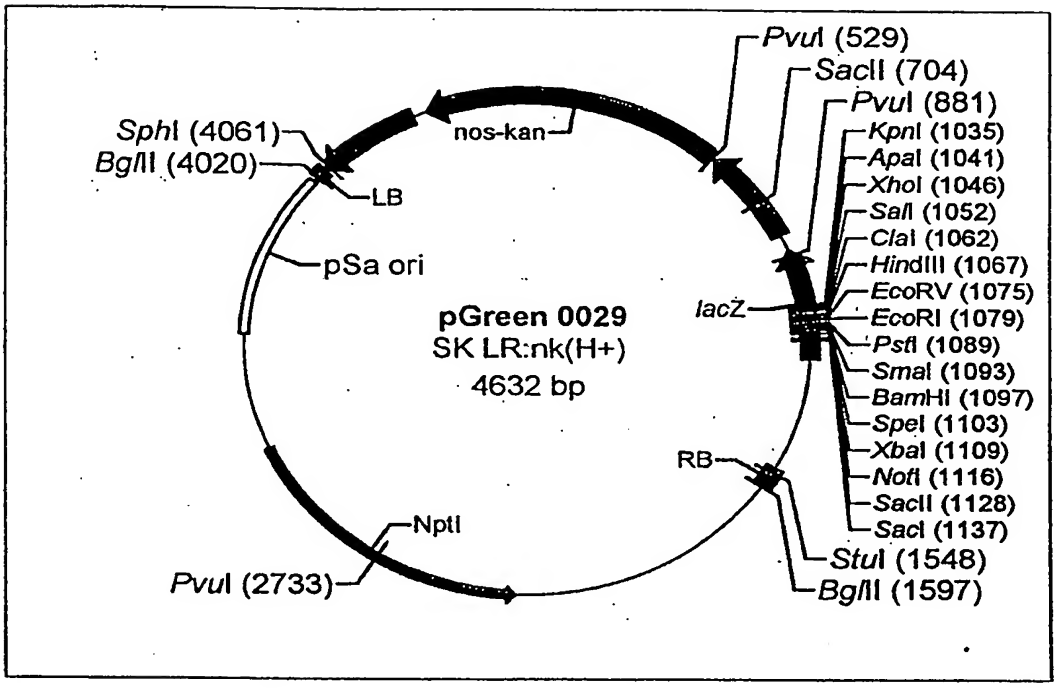
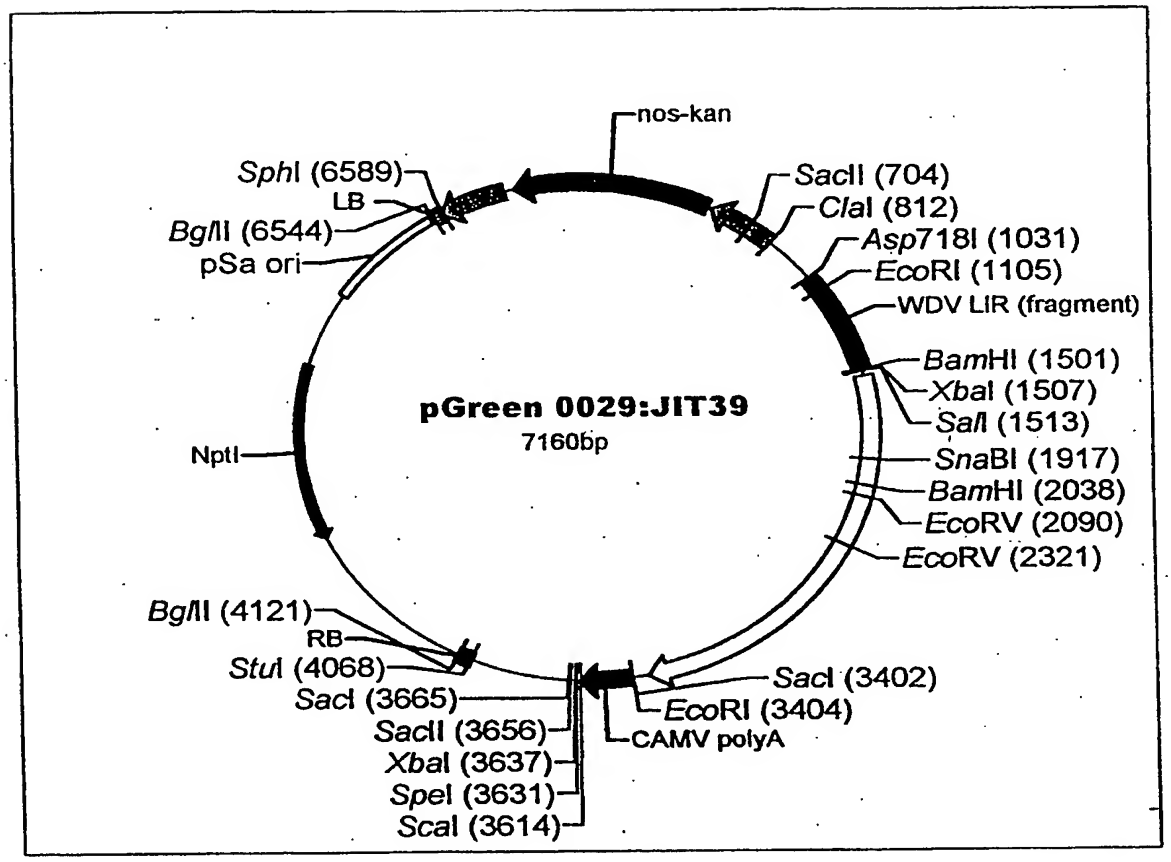
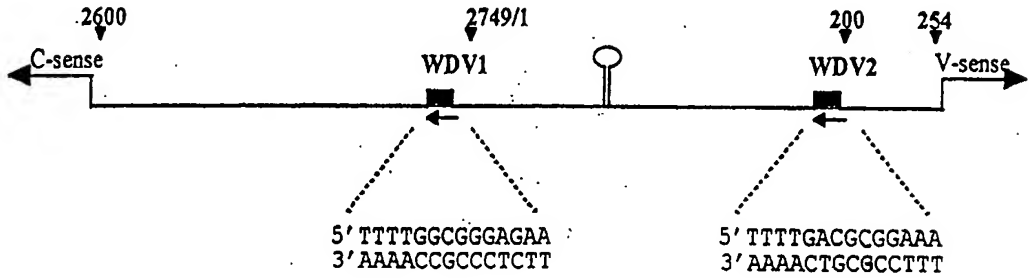

PvuI (529)
SacII (704)
PvuI (881)
KpnI (1035)
ApaI (1041)
XhoI (1046)
SalI (1052)
ClaI (1062)
HindIII (1067)
EcoRV (1075)
EcoRI (1079)
PstI (1089)
SmaI (1093)
BamHI (1097)
SpeI (1103)
XbaI (1109)
NotI (1116)
SacII (1128)
SacI (1137)
StuI (1548)
BglII (1597)
SphI (4061)
BglII (4020)
nos-kan
LB
pSa ori
pGreen 0029
SK LR:nk(H+)
4632 bp
lacZ
RB
NptII
PvuI (2733)

Figure 5B


nos-kan
SphI (6589)
LB
BglII (6544)
pSa ori
SacII (704)
ClaI (812)
Asp718I (1031)
EcoRI (1105)
WDV LIR (fragment)
BamHI (1501)
XbaI (1507)
SalI (1513)
SnaBI (1917)
BamHI (2038)
EcoRV (2090)
EcoRV (2321)
pGreen 0029:JIT39
7160bp
NptII
BglII (4121)
RB
StuI (4068)
SacI (3665)
SacII (3656)
XbaI (3637)
SpeI (3631)
ScaI (3614)
SacI (3402)
EcoRI (3404)
CAMV polyA

A



B

WDV-1	TTCTCCCGCCAAAA
WDV-2	TTTCCGCGTCAAAA
Core	TTTCGCGC
E2F class I	TTTTCCCGCCAAAA
E2F class II	TTTTCCCGCCTTTTTT
E2F class III	TTTTCCCGCGCTTTTTT
E2F class IV	TTTTCCCGCGCAAAA
E2A	TTTTCGCGCAATT
	TTTTCGCGCTTAAA
	TTTCGCGCCCTTT
E1A	TTTTCGCGCGGTTTT
	TTTTCCCGCGAAAA
c-myc	TTTTTCCCGCCAA
DHFR	TTTCGCGCCAAA

Figure 6

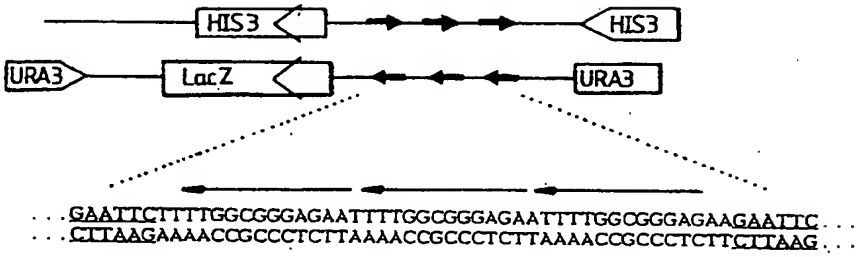


Fig. 7A

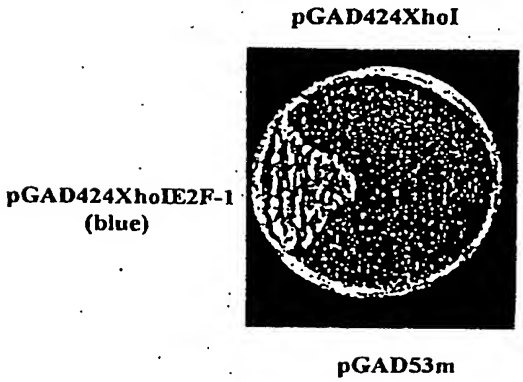


Fig. 7B

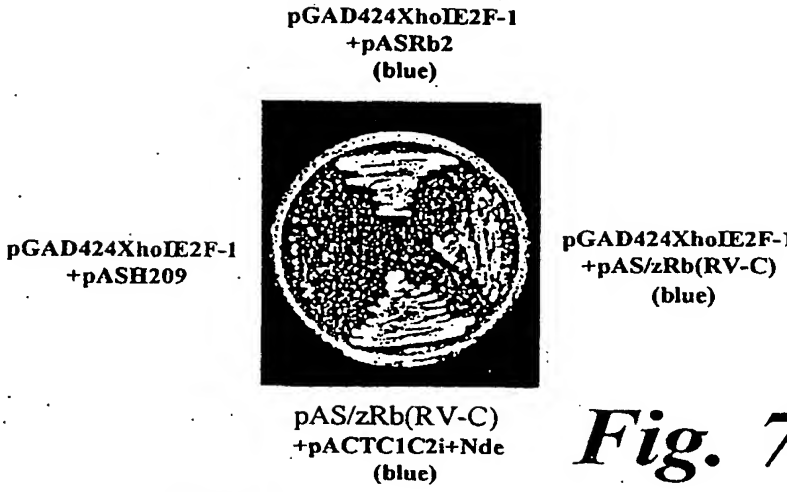


Fig. 7C

Figure 8

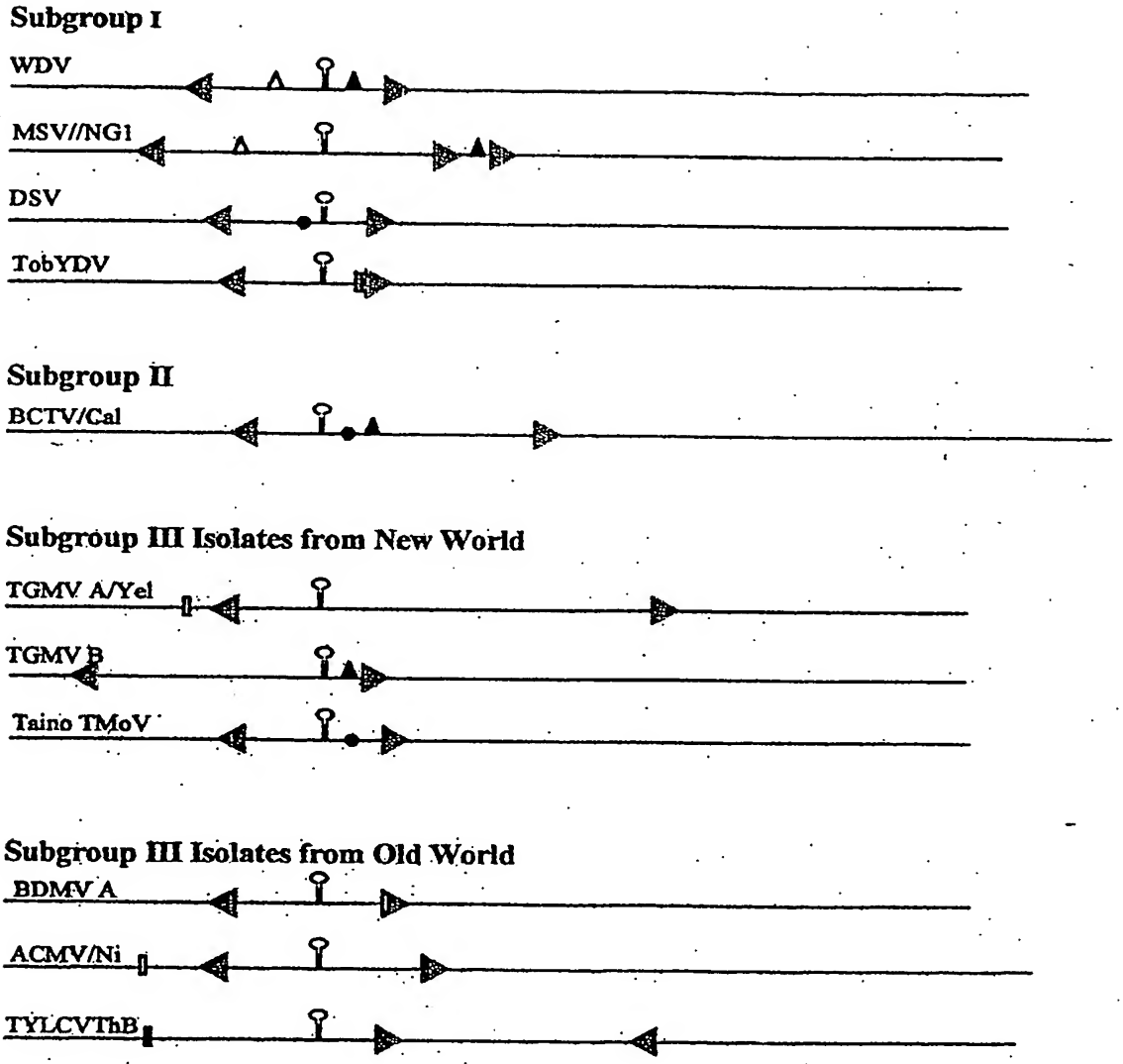

Subgroup I
WDV
MSV//NG1
DSV
TobYDV
Subgroup II
BCTV/Cal
Subgroup III Isolates from New World
TGMV A/Yel
TGMV B
Taino TMoV
Subgroup III Isolates from Old World
BDMV A
ACMV/Ni
TYLCVThB

Figure 9

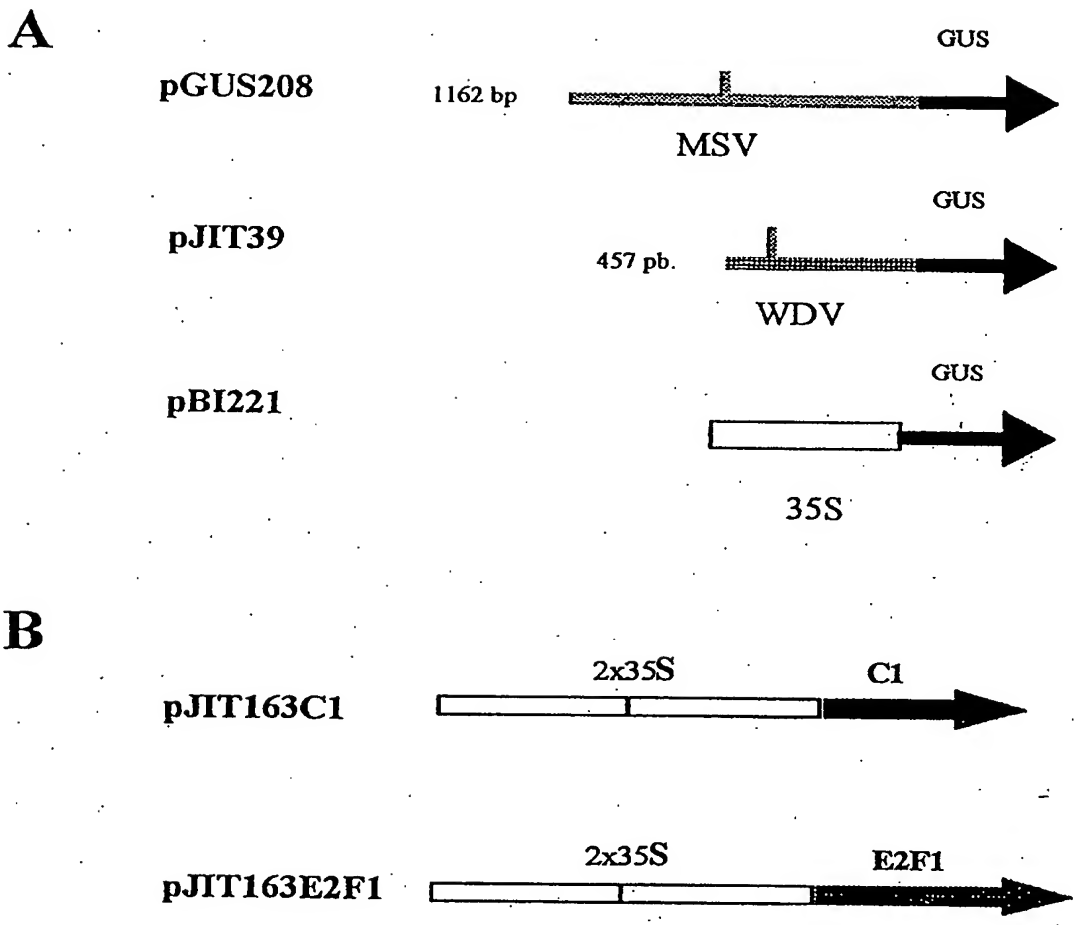
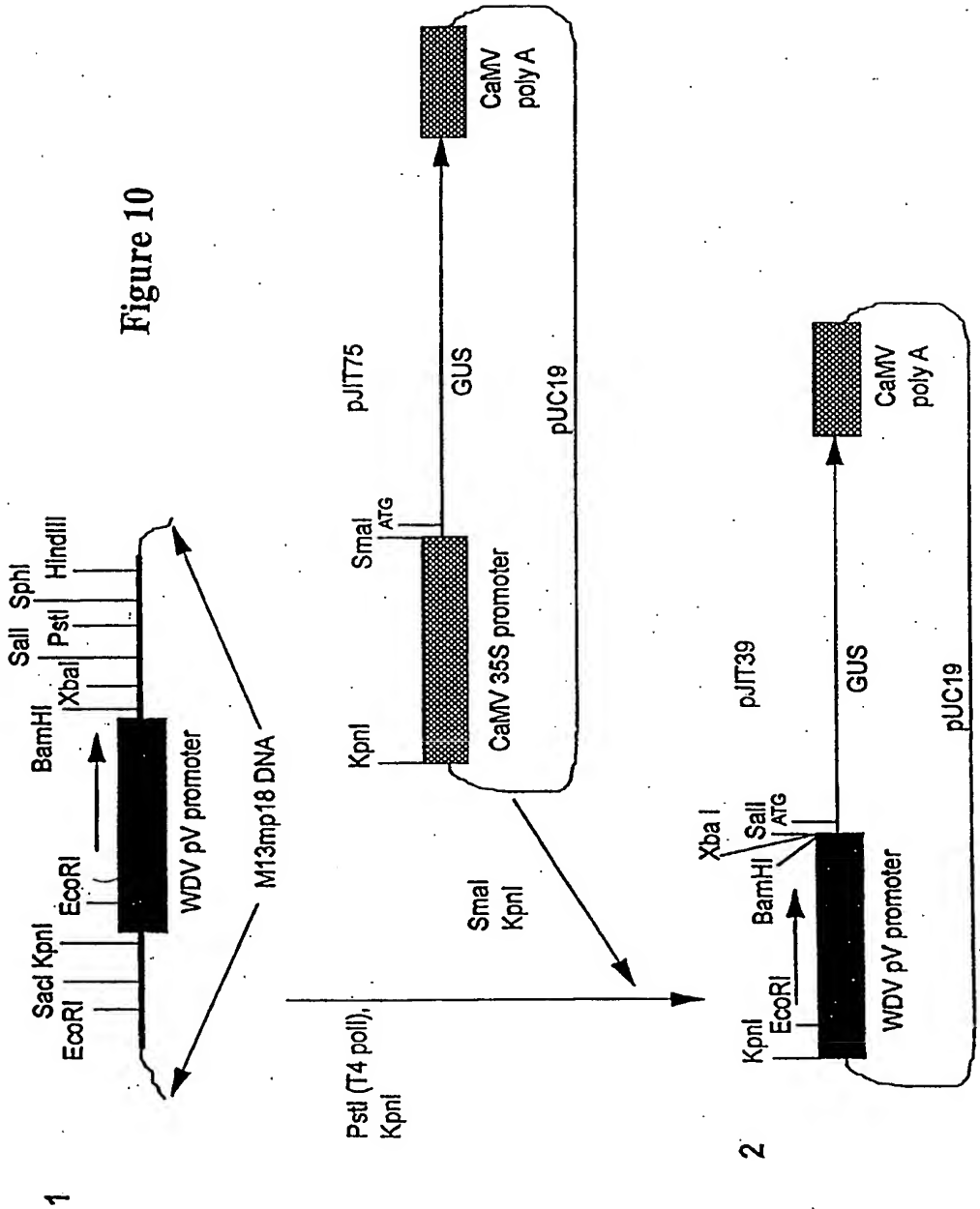

A
pGUS208
1162 bp
GUS
MSV
pJIT39
457 pb.
GUS
WDV
pBI221
GUS
35S
B
pJIT163C1
2x35S
C1
pJIT163E2F1
2x35S
E2F1

Figure 10


1
SacI KpnI
EcoRI
EcoRI
BamHI
XbaI
SalI
PstI
SphI
HindIII
WDV pV promoter
M13mp18 DNA
PstI (T4 polI),
KpnI
KpnI
SmaI
ATG
pJIT75
GUS
CaMV 35S promoter
pUC19
CaMV
poly A
SmaI
KpnI
2
KpnI
EcoRI
BamHI
Xba I
SalI
ATG
WDV pV promoter
pJIT39
GUS
pUC19
CaMV
poly A

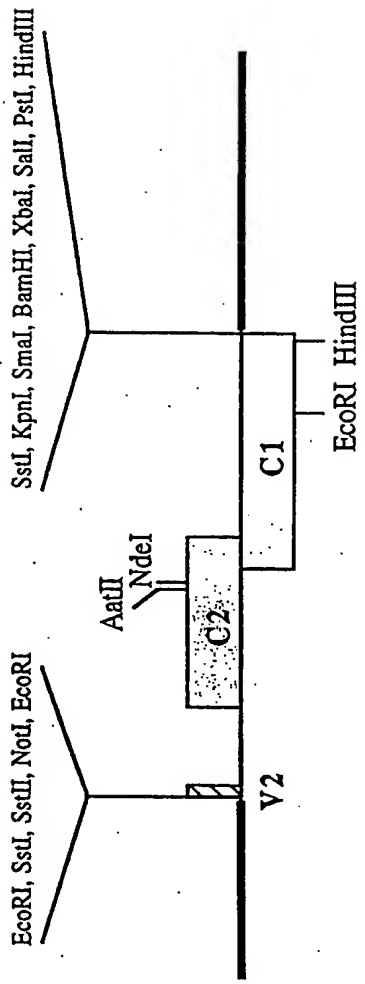

SstI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, HindIII
EcoRI, SstI, SstII, NotI, EcoRI
AatII
NdeI
C2
C1
V2
EcoRI HindIII
0.5 kilobases

Figure 11A

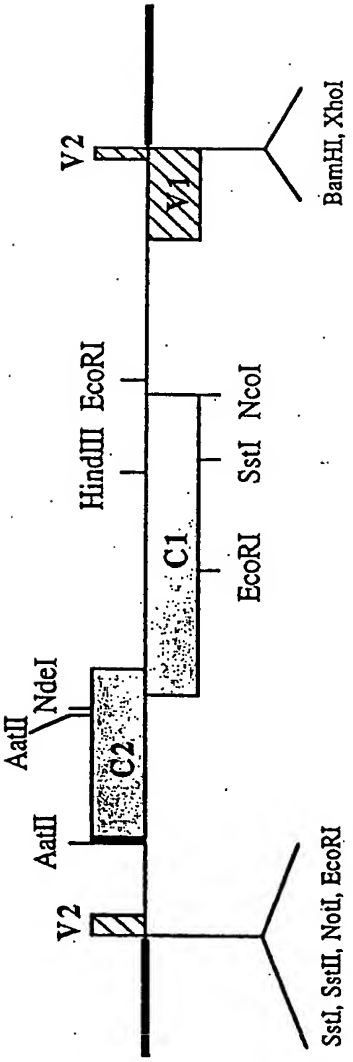

V2
BamHI, XhoI
HindIII EcoRI
SstI NcoI
EcoRI
C1
AatII
NdeI
C2
AatII
V2
SstI, SstII, NotI, EcoRI
0.5 kilobases

Figure 11B

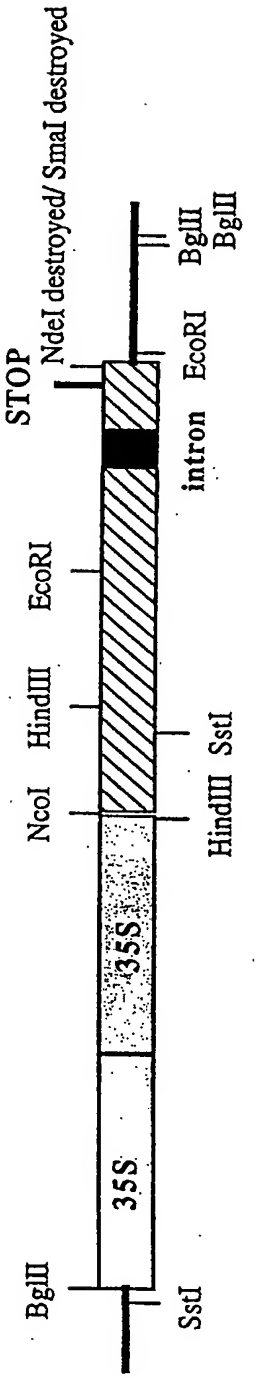

STOP
NdeI destroyed/ SmaI destroyed
BglII
BglII
EcoRI
intron
EcoRI
HindIII
NcoI
HindIII
SstI
35S
35S
BglII
SstI
0.5 kilobases

Figure 11C

Figure 12

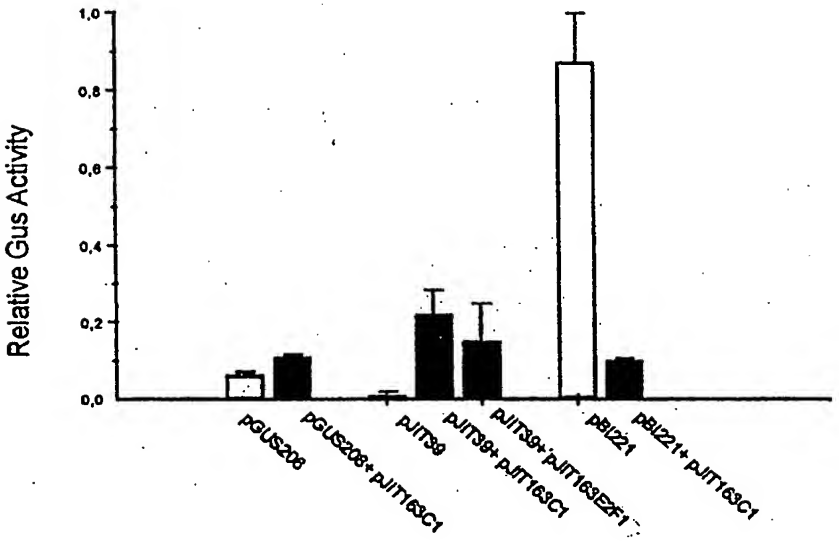

Relative Gus Activity
1,0
0,8
0,6
0,4
0,2
0,0
pGUS208
pGUS208+pJIT163C1
pJIT39
pJIT39+pJIT163C1
pJIT39+pJIT163E2F1
pBI221
pBI221+pJIT163C1

Fig. 13A

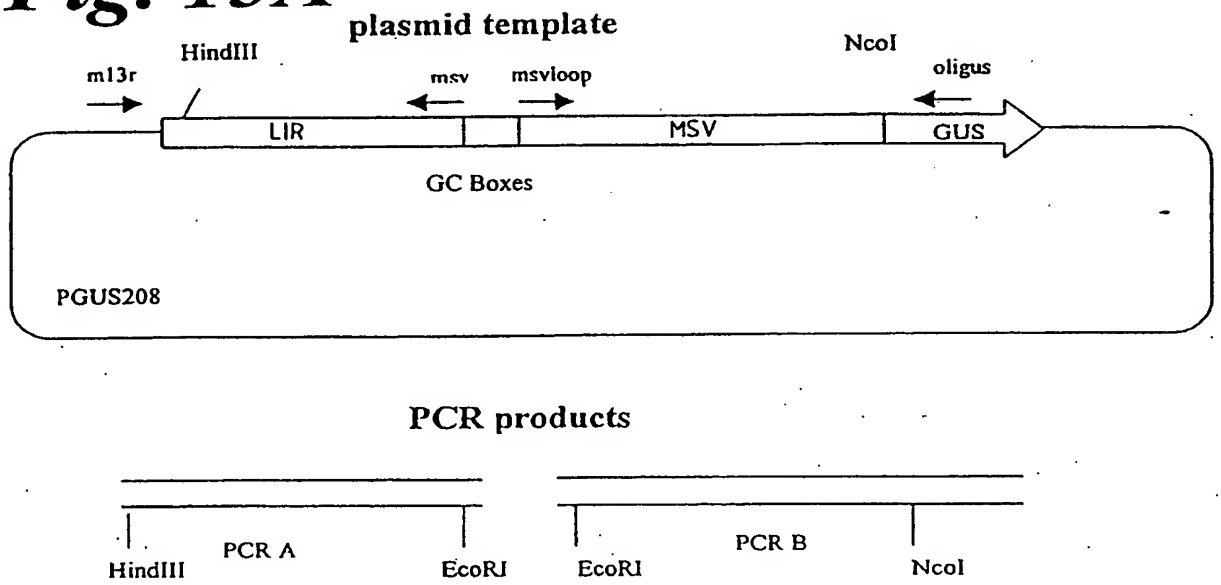


Fig. 13B

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TGGATCATGGAGAGGTACTCTTCTTTGGAAGTAGCGTGTGAAATAATGTCTCGCATTATTTCAT
CTTTAGAAGGCTTTTTTTCCTTTACCTCTGAATCAGATTTTCCGAGGAAGGGGGACTTCCTAGG
AATGAAAGTACCTCTCTCAAACACAGCCAGAGGTTCCTTGAGAATGTAATCCCTCACCCTGTTT
ACTGACTTGGCACTCTGAATATTTGGGTGAAACCCATTTATATCAAAGAACCTTGAGTCAGATA
TCCTTACCGGCTTCTCTGTCTGAAGCAATGCATGTAAATGCAAACTTCCATCTTTATGTGCCTC
TCGGGCACATAGAATGTATTTGGGAATCCAACGAACAACGAGCTCCCAGATCATCTGACAGGCG
ATTTCAGGATTTTCTGGACACTTTGGATAGGTTAGGAACGTGTTAGCGTTCCGGTGTGAGAACT
GACGGTTGGATGAGGAGGAGGCCATTGCCGACGACGGAGGTTGAGGCTGAGGGATGGCAGACTG
GGAGCTCCAAACTCTATAGTATACCCGTGCGCCTTCGCCTCGAGGCGAAATCCGCCGCTCCCTT
GTCTTGTAGTGGTTGCAAATGAATTCAGCAGGAAAAGAAGGCGCGCACTAATATTACCGCGCCT
TCTTTTCCTGCGAGGGCCCGGTAGGGTCGACCCCGAGCGATTTGATGTAAAGTTTGGTCCTGCT
TTGTATGATTTATCTAAAGCAGCCCATTCTAAAGAATCCGGTCCCGGTCACTATAAATTGCCTA
ACAAGTGCGATTCATTCATGGATCCACAGAACGCCCTGTATTATCAGCCGCGGGTACCCACAGC
AGCTCCGACATCCGGAGGAGTGCCGTGGAGTCGCGTAGGCGAGGTAGCTATTTTGAGCTTTGTT
GCATTGATTTGCTTTTACCTGCTTTACCTTTGGGTGCTGAGAGACCTTATCTTAGTTCTGAAGG
CTCGACAAGGCAGATCCACGGAGGAGCTGATATTTGGTGGACAAGCTGTGGATAGGAGCAACCC
TATCCCTAATATACCAGCACCACCAAGTCAGGGCAATCCCGGGCCATTTGTTCCATCGACTCTA
GTCGACCATGG

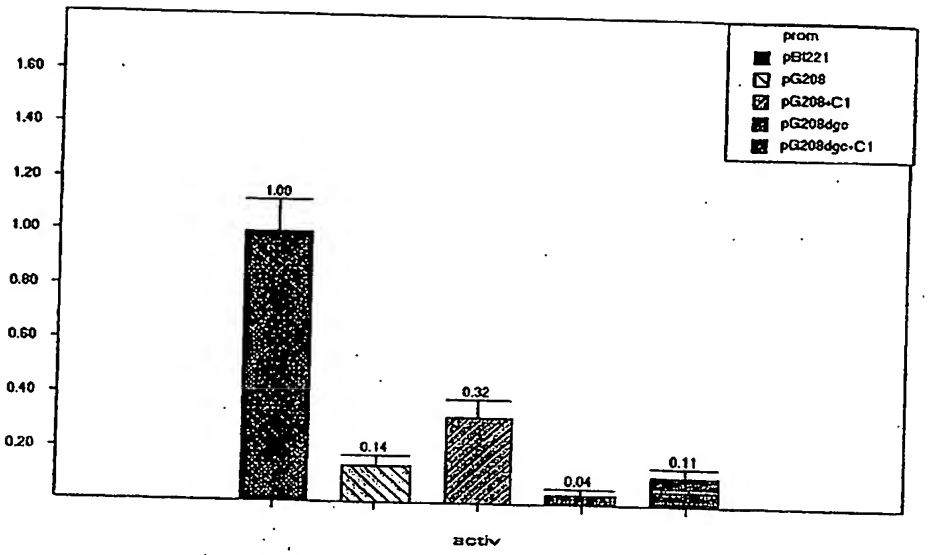
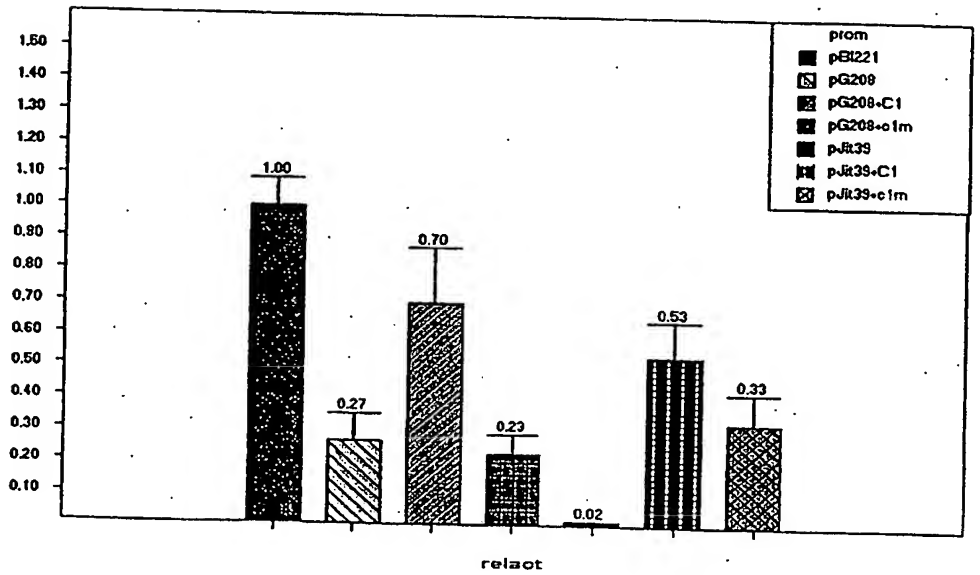

prom
pBt221
pG208
pG208+C1
pG208dgc
pG208dgc+C1
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
1.00
0.14
0.32
0.04
0.11
activ

Figure 16


prom
pBt221
pG208
pG208+C1
pG208+c1m
pJit39
pJit39+C1
pJit39+c1m
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
1.00
0.27
0.70
0.23
0.02
0.53
0.33
relact

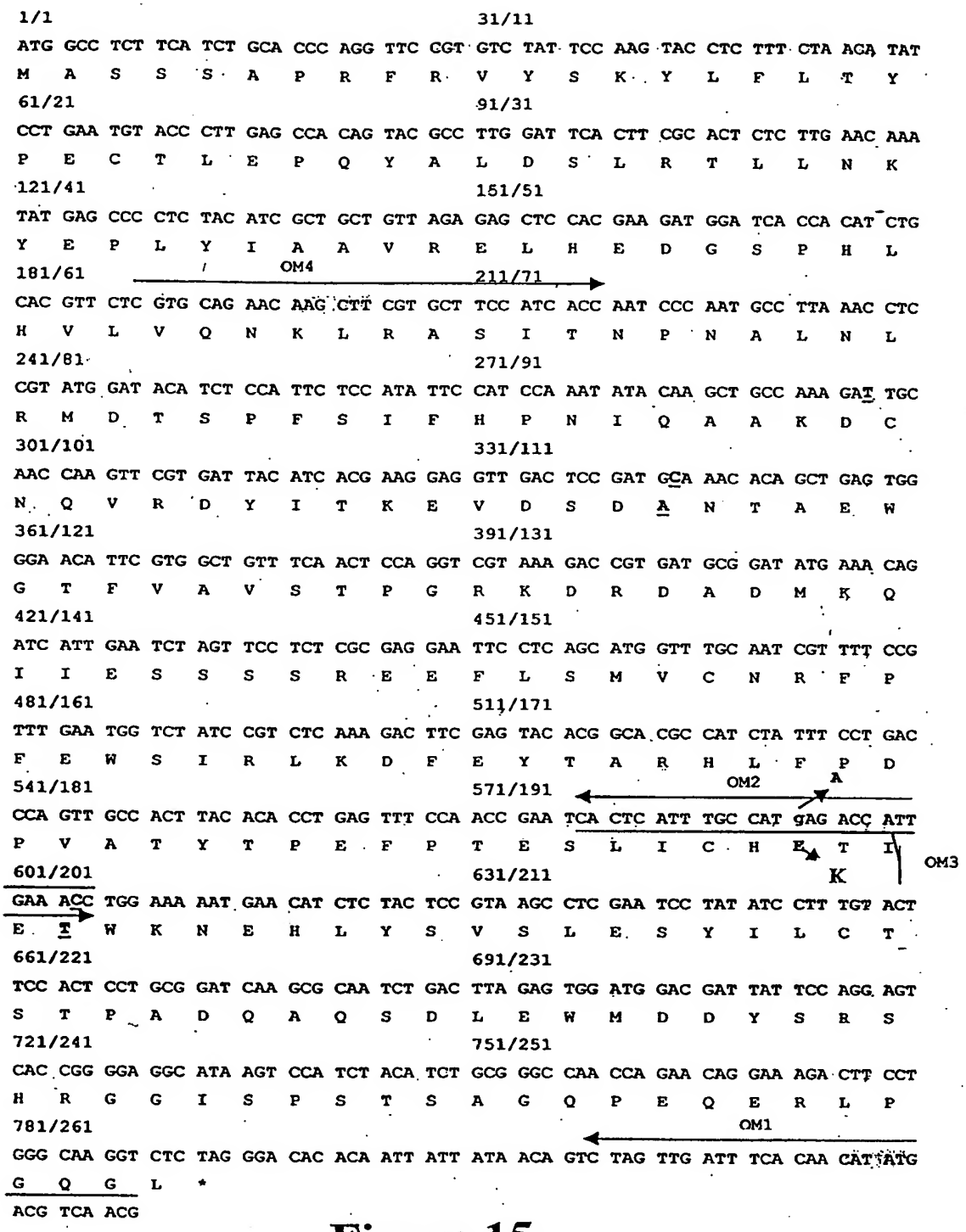

1/1
31/11
ATG GCC TCT TCA TCT GCA CCC AGG TTC CGT GTC TAT TCC AAG TAC CTC TTT CTA AGA TAT
M A S S S A P R F R V Y S K Y L F L T Y
61/21
91/31
CCT GAA TGT ACC CTT GAG CCA CAG TAC GCC TTG GAT TCA CTT CGC ACT CTC TTG AAC AAA
P E C T L E P Q Y A L D S L R T L L N K
121/41
151/51
TAT GAG CCC CTC TAC ATC GCT GCT GTT AGA GAG CTC CAC GAA GAT GGA TCA CCA CAT CTG
Y E P L Y I A A V R E L H E D G S P H L
181/61
OM4
211/71
CAC GTT CTC GTG CAG AAC AAG CTT CGT GCT TCC ATC ACC AAT CCC AAT GCC TTA AAC CTC
H V L V Q N K L R A S I T N P N A L N L
241/81
271/91
CGT ATG GAT ACA TCT CCA TTC TCC ATA TTC CAT CCA AAT ATA CAA GCT GCC AAA GAT TGC
R M D T S P F S I F H P N I Q A A K D C
301/101
331/111
AAC CAA GTT CGT GAT TAC ATC ACG AAG GAG GTT GAC TCC GAT GCA AAC ACA GCT GAG TGG
N Q V R D Y I T K E V D S D A N T A E W
361/121
391/131
GGA ACA TTC GTG GCT GTT TCA ACT CCA GGT CGT AAA GAC CGT GAT GCG GAT ATG AAA CAG
G T F V A V S T P G R K D R D A D M K Q
421/141
451/151
ATC ATT GAA TCT AGT TCC TCT CGC GAG GAA TTC CTC AGC ATG GTT TGC AAT CGT TTT CCG
I I E S S S S R E E F L S M V C N R F P
481/161
511/171
TTT GAA TGG TCT ATC CGT CTC AAA GAC TTC GAG TAC ACG GCA CGC CAT CTA TTT CCT GAC
F E W S I R L K D F E Y T A R H L F P D
541/181
571/191
OM2
A
CCA GTT GCC ACT TAC ACA CCT GAG TTT CCA ACC GAA TCA CTC ATT TGC CAT GAG ACC ATT
P V A T Y T P E F P T E S L I C H E T I
K
OM3
601/201
631/211
GAA ACC TGG AAA AAT GAA CAT CTC TAC TCC GTA AGC CTC GAA TCC TAT ATC CTT TGT ACT
E T W K N E H L Y S V S L E S Y I L C T
661/221
691/231
TCC ACT CCT GCG GAT CAA GCG CAA TCT GAC TTA GAG TGG ATG GAC GAT TAT TCC AGG AGT
S T P A D Q A Q S D L E W M D D Y S R S
721/241
751/251
CAC CGG GGA GGC ATA AGT CCA TCT ACA TCT GCG GGC CAA CCA GAA CAG GAA AGA CTT CCT
H R G G I S P S T S A G Q P E Q E R L P
781/261
OM1
GGG CAA GGT CTC TAG GGA CAC ACA ATT ATT ATA ACA GTC TAG TTG ATT TCA CAA CAT ATG
G Q G L *
ACG TCA ACG

Figure 15